

# Transient calnexin interaction confers long-term stability on folded K<sup>+</sup> channel protein in the ER

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## Summary

We recently showed that an unglycosylated form of the Shaker potassium channel protein is retained in the endoplasmic reticulum (ER) and degraded by proteasomes in mammalian cells despite apparently normal folding and assembly. These results suggest that channel proteins with a native structure can be substrates for ER-associated degradation. We have now tested this hypothesis using the wild-type Shaker protein. Wild-type Shaker is degraded by cytoplasmic proteasomes when it is trapped in the ER and prevented from interacting with calnexin. Neither

condition alone is sufficient to destabilize the protein. Proteasomal degradation of the wild-type protein is abolished when ER mannosidase I trimming of the core glycan is inhibited. Our results indicate that transient interaction with calnexin provides long-term protection from ER-associated degradation.

Key words: Potassium channels, Calnexin, ERAD, Proteasome, Glycan trimming, ER glucosidase/mannosidase, Shaker, Voltage-dependent, Glycosylation

## Introduction

Biogenesis of membrane and secretory proteins occurs in the endoplasmic reticulum (ER) (Hegde and Lingappa, 1999; Deutsch, 2002). This compartment contains a stringent quality control system that recognizes and retains proteins that fail to fold or assemble properly, thereby preventing the trafficking of aberrant proteins to cellular destinations where abnormal functional properties could disrupt cellular physiology (Kopito, 1997; Brodsky and McCracken, 1999; Zerangue et al., 1999; Ellgaard and Helenius, 2001; Helenius and Aebi, 2001; Deutsch, 2002). In the absence of forward trafficking, many ER-retained proteins are substrates for ER-associated degradation (ERAD) (Lippincott-Schwartz et al., 1988). Typically, ERAD substrates are dislocated from the ER for degradation by cytoplasmic proteasomes (Ward et al., 1995; Hiller et al., 1996; Yang et al., 1998; Zhou et al., 1999; Kopito, 2000).

Although most ERAD substrates are thought to be targeted for degradation because they are misfolded, we have recently shown that an unglycosylated form of the Shaker potassium channel protein is largely retained in the ER and degraded by proteasomes in mammalian cells despite apparently normal folding and assembly (Khanna et al., 2001). From these results, we hypothesized that channel proteins with a native structure can be substrates for ERAD. To test this hypothesis, we have now sought to identify conditions that lead to ERAD of a properly folded channel protein, the Shaker wild-type protein.

The Shaker channel is the prototype of a large superfamily of voltage-gated potassium channels (Papazian, 1999). Potassium channels control the excitability of nerve and muscle and also play crucial physiological roles in nonexcitable cells, where they maintain the negative resting membrane potential required for calcium influx during cell

signaling (Leonard et al., 1992; Kalman et al., 1998; Beeton et al., 2001). Potassium channels contain four transmembrane  $\alpha$  subunits that surround a central water-filled pore for K<sup>+</sup> conduction (MacKinnon, 1991; Doyle et al., 1998). Each  $\alpha$  subunit has six transmembrane segments, S1-S6, and a re-entrant loop that forms the narrowest part of the pore (Jan and Jan, 1997). It is not clear how the structure of a potassium channel is formed during biogenesis. However, it is known that disruption of channel biogenesis can result in human diseases, including long QT syndrome, a disorder characterized by ventricular arrhythmia resulting from slowed repolarization of the cardiac action potential, and episodic ataxia type 1, a neurological syndrome characterized by myokymia and intermittent ataxia (Nerbonne, 2000; Splawski et al., 2000; Manganas et al., 2001; Rea et al., 2002).

The Shaker protein provides an excellent model for studying potassium channel biogenesis and quality control (Papazian, 1999). The folding, assembly and maturation of the wild-type protein occur rapidly and efficiently in diverse expression systems (Schulzeis et al., 1996; Schulzeis et al., 1998; Papazian, 1999). This results in robust expression of stable, functional channels on the cell surface. By contrast, misfolded or unassembled mutant forms of the Shaker protein are efficiently recognized by the quality control system of the ER, leading to their intracellular retention and eventual disposal (Schulzeis et al., 1998).

Importantly, a biochemical hallmark of the native Shaker structure has been identified, providing a way to assess the structural integrity of mutant proteins that fail to traffic to the cell surface (Schulzeis et al., 1996; Schulzeis et al., 1998). Two cytoplasmic cysteine residues, one in the amino terminus (C96) and one in the carboxyl terminus (C505) of adjacent subunits, come into proximity during biogenesis (Schulzeis et al., 1998).

On treatment of intact cells with mild oxidants, C96 and C505 form an intersubunit disulfide bond (Schulteis et al., 1996). This bond can be oxidized efficiently in the wild-type protein, whether it is located at the plasma membrane or in the ER, and in mutant proteins that achieve the native structure, as indicated by maturation and functional activity (Schulteis et al., 1996; Nagaya and Papazian, 1997; Schulteis et al., 1998). However, in a variety of ER-retained mutant proteins, the C96/C505 bond cannot be oxidized (Schulteis et al., 1998). These results suggest that the ability to form the C96/C505 bond is restricted to Shaker proteins that are able to fold and assemble properly. This interpretation is supported by the results of second site suppressor analysis (Tiwari-Woodruff et al., 1997). We have presented evidence that the mutations D316K in S3 and K374E in S4 prevent proper folding of the voltage sensor by disrupting an electrostatic interaction between these positions (Tiwari-Woodruff et al., 1997). The D316K and K374E proteins are retained in the ER and are unable to form the C96/C505 disulfide bond (Schulteis et al., 1998). On combination of these two mutations in a single subunit, however, maturation and functional activity are restored, providing strong evidence that the double-mutant protein folds and assembles properly (Tiwari-Woodruff et al., 1997). Concomitantly, the ability to form the C96/C505 disulfide bond is restored in the D316K+K374E double-mutant protein (Schulteis et al., 1998).

In a recent study, we investigated the role of *N*-linked glycosylation in the stability, surface expression and folding of Shaker K<sup>+</sup> channels (Khanna et al., 2001). The Shaker protein is glycosylated at two asparagine residues, N259 and N263, located in the extracellular loop between the first and second transmembrane segments (Santacruz-Toloza et al., 1994). An unglycosylated mutant protein, N259Q+N263Q, was expressed in HEK293T cells. Much of the N259Q+N263Q protein was retained intracellularly, in contrast to the robust surface expression of the wild type (Khanna et al., 2001). The wild-type protein was extremely stable, whereas N259Q+N263Q was degraded by cytoplasmic proteasomes with a half-time of approximately 18 hours. We assessed the structural integrity of the N259Q+N263Q protein using the C96/C505 disulfide bond assay (Schulteis et al., 1996). This disulfide bond formed with equal efficiency in the wild-type and N259Q+N263Q proteins (Khanna et al., 2001). These results suggest that glycosylation increases the stability and cell-surface expression of Shaker protein, but has little effect on acquisition of the native structure.

If the N259Q+N263Q protein does in fact fold and assemble properly, as suggested by the C96/C505 disulfide bond folding assay, then it should be possible to identify conditions that lead to ERAD of the wild-type Shaker protein. What factors might account for the differential stability of wild-type and N259Q+N263Q? One possibility is ER exit rate. The wild-type protein exits the ER efficiently, whereas N259Q+N263Q is largely retained in that compartment (Khanna et al., 2001). A second possibility is *N*-linked glycosylation. The wild-type protein is glycosylated, whereas N259Q+N263Q is not. Glycan modification alters not only the physical properties of a protein, but also its ability to interact with the lectin chaperone, calnexin. We have previously shown that, soon after its synthesis, the wild-type Shaker protein interacts transiently with calnexin (Nagaya et al., 1999). This interaction requires glycosylation; N259Q+N263Q does not associate with calnexin (Nagaya et al., 1999).

Emerging evidence indicates that *N*-linked glycosylation and trimming of the core glycan in the ER play key roles in membrane protein biogenesis, ER retention and ERAD (Parodi, 1998; Spiro, 2000; Cabral et al., 2001; Roth et al., 2002). *N*-linked glycosylation begins with the co-translational addition of a preformed 14-residue core glycan to lumenally exposed asparagine residues that are part of the consensus sequence Asn-X-Ser/Thr (Helenius and Aebi, 2001). Processing of the core glycan begins immediately with trimming of two of the three terminal glucose residues by ER glucosidases I and II. Generation of the monoglucosylated core glycan results in the association of the newly made glycoprotein with calnexin and/or calreticulin. These homologous lectins are part of an ER chaperone system that also includes ER glucosidase II and UDP-glucose:glycoprotein glucosyltransferase. These enzymes catalyze a cycle of deglycosylation and reglycosylation while the protein acquires its native structure. Properly folded molecules are released from this cycle, whereas misfolded molecules are retained in the ER, where they may become ERAD substrates (Helenius and Aebi, 2001). For some proteins, interaction with calnexin has been shown to inhibit or delay ERAD (Keller et al., 1998; de Virgilio et al., 1999; Wang and White, 2000; Wilson et al., 2000). By contrast, further processing of the core glycan, specifically trimming of a particular mannose residue by ER mannosidase I, has been shown to promote proteasomal degradation of several glycoproteins (Liu et al., 1997; Jakob et al., 1998; Yang et al., 1998; de Virgilio et al., 1999; Liu et al., 1999; Chung et al., 2000; Wang and White, 2000; Tokunaga et al., 2000; Fagioli and Sitia, 2001).

In this study, we sought to identify conditions that result in ERAD of properly folded and assembled K<sup>+</sup> channel protein. We report that transient interaction with calnexin confers long-term protection from degradation on the wild-type Shaker protein localized in the ER. In the absence of calnexin interaction and ER retention, however, efficient ER exit spares the wild-type protein from degradation, presumably by separating it from the ERAD targeting machinery.

## Materials and Methods

### Materials

Brefeldin A (BFA) and nocodazole (NOC) were purchased from Sigma (St Louis, MO). Clasto-lactacystin- $\beta$ -lactone, the active form of the proteasome inhibitor, lactacystin (LAC), was from Calbiochem (La Jolla, CA). Deoxynojirimycin (dNJ), deoxymannojirimycin (dMJ), kifunensine (KIF) and swainsonine (SWN) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Antibodies directed against a Shaker- $\beta$ -galactosidase fusion protein were the generous gift of L.Y. Jan (University of California, San Francisco) (Schwarz et al., 1990). Antibodies directed against calnexin were purchased from Sigma (St Louis, MO) for immunoprecipitation and from Affinity Bioreagents (Golden, CO) for immunofluorescence. Antibodies directed against calreticulin were purchased from Novus Biologicals (Littleton, CO).

### Cell culture and metabolic labeling, immunoprecipitation, electrophoresis and fluorography

Culturing of human embryonic kidney 293 cells (HEK293T), transient transfection, metabolic labeling, immunoprecipitation, electrophoresis and fluorography were performed as described previously (Khanna et al., 2001). Forty eight hours after transfection,

cells were incubated for 30 minutes in methionine- and cysteine-free Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA), pulsed with 0.2-1 mCi/ml [<sup>35</sup>S]-methionine and [<sup>35</sup>S]-cysteine (Tran<sup>35</sup>S-Label, ICN, Irvine, CA) for 2, 10 or 30 minutes, as indicated in the text; and chased for various times in complete, nonradioactive medium. Cells subjected to lactacystin (10 μM) treatment were pre-incubated for 12-16 hours with the drug, which was also present throughout the pulse and chase periods. Incubation with lactacystin had no apparent effect on cell growth; untreated and lactacystin-treated cultures were confluent at the time of harvest. Cells were incubated with other drugs for 1-2 hours before the start of metabolic labeling and during the pulse and chase periods, except for post-translational dNJ treatment, where the drug was added to the chase period only.

The association of wild-type protein with calnexin was assayed as described previously (Nagaya et al., 1999), using a 2 or 5 minute pulse for metabolic labeling.

For endo H digestion, immunoprecipitated protein was boiled for 3 minutes in endo H buffer (50 mM sodium acetate, 10 mM EDTA, 0.06% SDS, 10 mM β-mercaptoethanol, pH 5.5) and then incubated for 16-18 hours at 37°C with 50 mUnits/ml endo H (Boehringer Mannheim, Indianapolis IN) in the presence of 0.36% Triton X-100, 0.5 mM phenylmethanesulfonate, 50 μg/ml antipain, 2 μg/ml aprotinin and 0.7 μg/ml pepstatin. The reaction was terminated by adding Laemmli sample buffer and boiling for 3 minutes (Nagaya and Papazian, 1997). PNGase F (Sigma, St Louis, MO) digestion was performed for 16-18 hours at 37°C with 5 Units/ml of enzyme in 100 mM NaCl, 1 mM CaCl<sub>2</sub>, 50 mM Tris, pH 8 (Santacruz-Tolozza et al., 1994; Schulteis et al., 1995).

For electrophoresis, each sample loaded was derived from an aliquot of cell lysate containing an equal number of trichloroacetic acid-precipitable counts per minute. Protein bands were quantified by densitometry (Personal Densitometer SI, Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA) using Molecular Dynamics ImageQuant software (v.4.2, Molecular Dynamics).

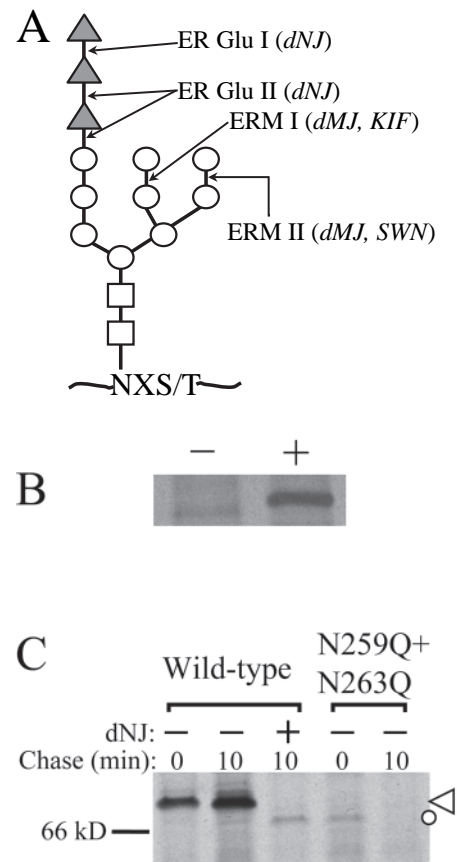
#### Immunofluorescence microscopy

Indirect immunofluorescence was performed as described previously (Khanna et al., 2001) using a polyclonal rabbit antiserum directed against a Shaker β-galactosidase fusion protein and a mouse monoclonal antibody against calnexin. Fluorescently conjugated secondary antibodies (Alexa-488-conjugated goat anti-rabbit [1:1000] and Alexa-568-conjugated goat anti-mouse [1:1500]) were used to visualize the proteins. Images were obtained with a 63× quartz objective on an inverted laser scanning confocal microscope (Leica Dm IRB/E, Meyer Instruments, Houston, TX).

## Results

### Interaction with calnexin increases ER exit rate

We first investigated whether the failure to interact with lectin chaperones was sufficient to cause ER retention and degradation of the properly folded wild-type Shaker protein. Previously, we showed that calnexin interacts transiently with wild-type Shaker soon after its synthesis. Following a 2 minute pulse, maximal association is seen after 10 minutes of chase (Nagaya et al., 1999). To prevent interaction with calnexin, Shaker-expressing cells were treated with deoxymannojirimycin (dNJ), an inhibitor of ER glucosidases I and II (Fig. 1A) (Helenius and Aebi, 2001). When present co-translationally, dNJ prevents the formation of the monoglucosylated core glycan recognized by calnexin and calreticulin, and thereby prevents association with these chaperones (Helenius and Aebi, 2001). As previously



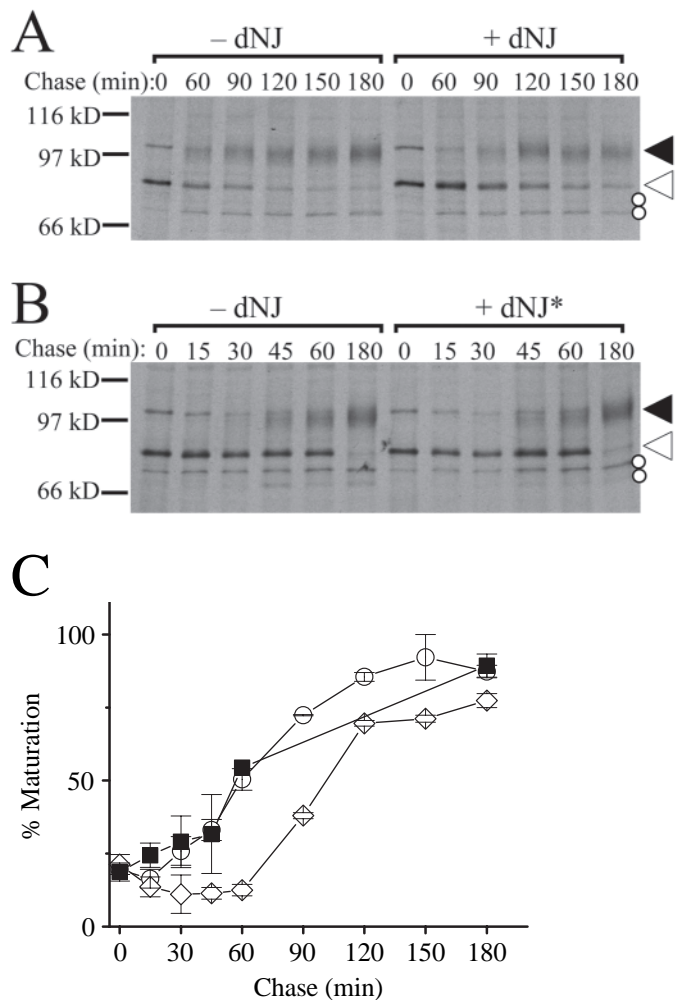
**Fig. 1.** Treatment with dNJ prevents association of the core glycosylated Shaker protein with calnexin. (A) Structure of the 14-residue core glycan is shown. Glucose (▲), mannose (○) and *N*-acetylglucosamine (□). Arrows indicate sites of trimming by ER glucosidases I and II (ER Glu I and II) and ER mannosidases I and II (ERMI and II). Drugs that inhibit each step are shown in parentheses: dMJ, deoxymannojirimycin; dNJ, deoxynojirimycin; KIF, kifunensine; SWN, swainsonine. (B) Adjacent lanes showing the mobility of the immature, core-glycosylated form of the Shaker protein from cells incubated in the absence (-) or presence (+) of dNJ. When present, dNJ was added before the starvation step and was present throughout the pulse and chase periods. (C) Cells expressing the wild-type Shaker protein or an unglycosylated mutant (N259Q+N263Q) were metabolically labeled for 5 minutes, chased in nonradioactive medium for 0 or 10 minutes, as noted, and lysed with detergent. When present (+), dNJ was added before the starvation step and was present throughout the pulse and chase periods. Proteins were precipitated sequentially with antibodies directed against calnexin and Shaker (Nagaya et al., 1999). The open arrowhead denotes the position of the immature form of the Shaker protein. The open circle indicates the position of a nonspecific background band of variable intensity, which is also seen in untransfected cells upon immunoprecipitation with the Shaker antiserum (data not shown).

reported, treatment with a glucosidase inhibitor resulted in a small but reproducible decrease in the mobility of the immature, core glycosylated form of the Shaker protein (Fig. 1B) (Nagaya et al., 1999). To verify that dNJ treatment prevented interaction between the Shaker protein and calnexin, wild-type Shaker was expressed in HEK293T cells, metabolically labeled during a 5 minute pulse, and either

harvested immediately or chased in nonradioactive medium for 10 minutes in the presence or absence of dNJ. When present, dNJ was added before the starvation period and maintained in the medium throughout the pulse and chase periods. The cells were then extracted with detergent, and solubilized material was subjected to immunoprecipitation with an antibody directed against calnexin. Subsequently, proteins bound to calnexin were dissociated and reprecipitated with an antibody directed against Shaker. As previously reported, calnexin interacted specifically with the immature form of the Shaker protein (Fig. 1C) (Nagaya et al., 1999). Interaction with calnexin required glycosylation. Calnexin failed to interact with the unglycosylated mutant protein, N259Q+N263Q (Fig. 1C) (Nagaya et al., 1999). As expected, continuous treatment with dNJ during and after translation abolished the association of the wild-type Shaker protein with calnexin (Fig. 1C).

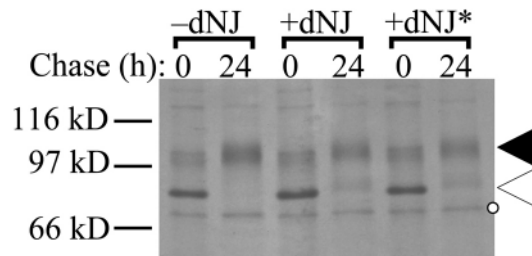
To determine whether the interaction with calnexin is required for subsequent ER exit, the wild-type Shaker protein was metabolically labeled during a 10 minute pulse, and then chased in nonradioactive medium for various times in the presence or absence of dNJ. dNJ was present either co- and post-translationally, as described above, or post-translationally only (drug added only during the chase period) (Fig. 2A,B). These treatments are expected to have different effects on the interaction between Shaker and calnexin (Helenius and Aebi, 2001). As shown above, treatment with dNJ during and after protein synthesis prevents the association with calnexin. By contrast, treatment during the chase period only can trap core glycans in the monoglucosylated form, prolonging interactions with calnexin (Helenius and Aebi, 2001); the 10 minute pulse period was chosen because it approximates the peak of association between Shaker and calnexin (Nagaya et al., 1999). It is worth noting that proper folding will ultimately release the protein from the calnexin chaperone system despite the presence of monoglucosylated core glycans (Helenius and Aebi, 2001).

As previously reported, in untreated cells the Shaker protein was detected after the pulse as a core glycosylated immature precursor that matured to the complex glycosylated mature form after processing in the *medial* Golgi compartment (Santacruz-Toloza et al., 1994; Schulteis et al., 1995; Nagaya and Papazian, 1997; Schulteis et al., 1998; Nagaya et al., 1999). Treatment with dNJ, either co- and post-translationally, or post-translationally only, did not prevent maturation or significantly reduce its extent (Fig. 2A,B). [The fact that complex glycosylation occurs despite the inhibition of ER glucosidases I and II by dNJ has been attributed to the activity of a Golgi-localized endomannosidase (Lubas and Spiro, 1988; Zuber et al., 2000).] These results indicate that failure to interact with calnexin does not result in ER retention of the Shaker protein. Interestingly, when present co-translationally, dNJ slowed the rate of maturation (Fig. 2C). This result suggests that interaction with calnexin increases the rate of ER exit. This conclusion is supported by the finding that dNJ added post-translationally had no effect on the rate of maturation (Fig. 2C). Thus, to slow ER exit, the drug must be added early in biogenesis to prevent association with calnexin. It is not surprising that post-translational addition of dNJ had no effect on Shaker maturation, because we have previously shown that the wild-type protein folds and assembles rapidly and



**Fig. 2.** Inhibition of glucose trimming slows ER exit rate of wild-type Shaker protein. (A,B) Wild-type Shaker protein was expressed in HEK293T cells, metabolically labeled for 10 minutes and chased for various times in the absence (-dNJ) or presence of 1 mM dNJ added during the starvation, pulse and chase periods (+dNJ) or during the chase period only (+dNJ\*). After detergent solubilization, the Shaker protein was immunoprecipitated and subjected to electrophoresis and fluorography. Representative fluorographs are shown;  $n=4$ . Open and closed arrowheads indicate the positions of the immature and mature forms of the Shaker protein, respectively. Open circles indicate the positions of two nonspecific background bands of variable intensity, which are also seen in untransfected cells upon immunoprecipitation with the Shaker antiserum (data not shown). A sharp, unstable band migrating near the mature form of the Shaker protein is also seen in untransfected cells (unmarked). Positions of molecular weight markers are indicated by bars (kD; kilodaltons). (C) The percentage of Shaker protein in the mature form has been plotted as a function of chase time. Data were obtained in the absence of dNJ ( $\circ$ ;  $t_{1/2}$  for maturation=59 minutes), in the presence of dNJ added during the starvation, pulse and the chase ( $\diamond$ ;  $t_{1/2}$ =101 minutes), or in the presence of dNJ added during the chase only ( $\blacksquare$ ;  $t_{1/2}$ =57 minutes) ( $n=4$ ). Here and in all subsequent figures, the data are presented as mean $\pm$ s.e.m.

efficiently, guaranteeing its timely release from the calnexin chaperone system (Schulteis et al., 1995; Schulteis et al., 1996; Nagaya and Papazian, 1997; Schulteis et al., 1998; Nagaya et al., 1999).



**Fig. 3.** Inhibition of glucose trimming does not destabilize the wild-type Shaker protein. After a 30 minute pulse, cells were harvested immediately (0 h) or after a 24 hour chase period. The amount of Shaker protein remaining at 24 hours was ~86% (-dNJ), ~81% (+dNJ) or ~81% (+dNJ\*). The parenthetical designations have the same meaning as in Fig. 2. Open and closed arrowheads indicate the positions of the immature and mature forms of the Shaker protein, respectively. The open circle indicates the position of a nonspecific background band of variable intensity, which is also seen in untransfected cells upon immunoprecipitation with the Shaker antiserum (data not shown). A representative fluorograph is shown ( $n=3$ ).

#### Failure to interact with calnexin does not destabilize Shaker wild-type protein

We next investigated whether failure to interact with lectin chaperones was sufficient to destabilize the wild-type Shaker protein. The protein was expressed in HEK293T cells, metabolically labeled during a 30 minute pulse, and either harvested immediately or after a 24 hour chase in nonradioactive medium. Cells were incubated in dNJ during the pulse and chase, during the chase only or were untreated with dNJ. As shown in Fig. 3, the wild-type protein was equally stable with or without treatment with dNJ.

We conclude that failure to interact with calnexin does not result in ER retention and degradation of properly folded Shaker protein. We further conclude that lack of association with calnexin is not sufficient to account for the ER retention and proteasomal degradation of the unglycosylated N259Q+N263Q protein (Khanna et al., 2001).

#### ER retention does not destabilize Shaker wild-type protein

We then tested the possibility that ER retention per se was sufficient to lead to proteasomal degradation of properly folded Shaker protein (Fig. 4). Cells expressing the wild-type Shaker protein were treated in the presence or absence of brefeldin A plus nocodazole (BFA/NOC) to prevent ER-to-Golgi transport (Nagaya and Papazian, 1997). Treatment with BFA/NOC prevented maturation of the Shaker protein, consistent with failure to traffic beyond the ER (Fig. 4A). Confocal microscopy confirmed that the Shaker protein was retained intracellularly in response to treatment with BFA/NOC, with substantial overlap with calnexin (Fig. 4B). By contrast, the wild-type Shaker protein was localized predominantly at the cell surface in the absence of BFA/NOC treatment (Fig. 4C). It is important to emphasize that the core-glycosylated wild-type protein folds and assembles properly in the ER when forward trafficking is prevented, as illustrated by the C96/C505 disulfide assay and the fact that maturation occurs rapidly and

efficiently when a reversible block of ER-to-Golgi transport is relieved (Nagaya and Papazian, 1997). Pulse-chase analysis revealed that the wild-type protein was stable, exhibiting little or no degradation at 24 and 48 hours of chase, whether allowed to traffic beyond the ER or retained in that compartment by BFA/NOC treatment (Fig. 4A). These results indicate that ER retention alone is not sufficient to destabilize the wild-type protein. The data also suggest that ER retention of the N259Q+N263Q protein is not responsible for its proteasomal degradation (Khanna et al., 2001).

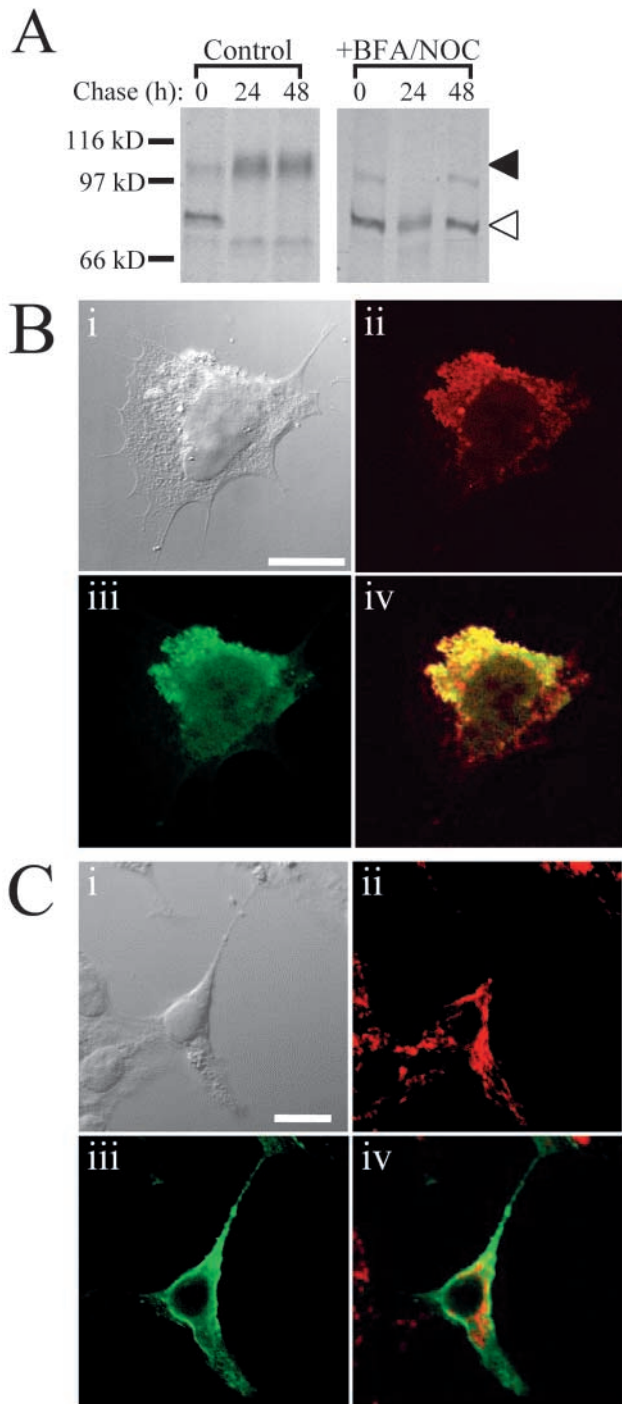
#### If ER exit is prevented, lack of interaction with calnexin results in proteasomal degradation

We next tested the hypothesis that a combination of ER retention and failure to interact with calnexin would result in ERAD of the wild-type Shaker protein. Cells were treated with BFA/NOC to trap wild-type Shaker in the ER, and in addition were incubated with dNJ, which was added during both the pulse and chase periods (Fig. 5). When dNJ was added, the wild-type protein (trapped in the ER) was significantly destabilized (Fig. 5A). At 48 hours of chase, only  $31\pm 5\%$  of the protein remained, compared with  $116\pm 22\%$  after BFA/NOC treatment alone (Fig. 5B). This result indicates that failure to interact with calnexin, coupled with ER localization, leads to the degradation of the wild-type Shaker protein.

To determine whether the wild-type protein was degraded by cytoplasmic proteasomes, the experiment was repeated in the presence of lactacystin (Fig. 5). When lactacystin was added in the presence of BFA/NOC and dNJ, degradation of the Shaker protein was inhibited. At 48 hours of chase,  $106\pm 24\%$  of the protein remained. This result indicates that the wild-type Shaker protein is targeted to proteasomes for degradation when retained in the ER and prevented from interacting with calnexin. These conditions recapitulate the fate of the N259Q+N263Q protein, and indicate that both ER retention and lack of interaction with calnexin contribute to the degradation of the unglycosylated mutant, N259Q+N263Q (Khanna et al., 2001).

#### Despite lack of calnexin interaction, wild-type protein matures on relief of ER-to-Golgi transport blockade

As shown above, the wild-type Shaker protein becomes a substrate for proteasomal degradation when it is trapped in the ER with BFA/NOC and prevented from interacting with calnexin by dNJ treatment (Fig. 5). To investigate whether the wild-type protein folds properly under these conditions, Shaker-expressing cells were treated with BFA/NOC and dNJ before and during a 30 minute pulse and a 2 hour chase. After 2 hours of chase, BFA/NOC was removed and the chase was continued in the presence of dNJ alone. Cells were harvested at various times, and Shaker protein was immunoprecipitated and subjected to electrophoresis. After removal of BFA/NOC, the core-glycosylated Shaker protein was converted with time to a larger, diffusely migrating band (Fig. 6A). This band was similar in appearance to the mature form of the protein observed in cells not subjected to drug treatment, although its mobility and micro-heterogeneity were somewhat increased compared with the untreated mature protein (Fig. 6A). To determine whether the diffuse band contained complex



carbohydrates, which are characteristic of the mature Shaker protein, its mobility was compared in the presence and absence of treatment with endoglycosidase H (endo H) (Fig. 6B). The diffusely migrating band was resistant to endo H digestion, in contrast to the remaining core glycosylated protein. As expected, the diffuse and core glycosylated proteins were both converted to a single band on removal of *N*-linked carbohydrates with PNGase F (Fig. 6B). These results indicate that, on removal of BFA/NOC in the continued presence of dNJ, the wild-type Shaker protein matured to a complex glycosylated form. Because wild-type Shaker remains

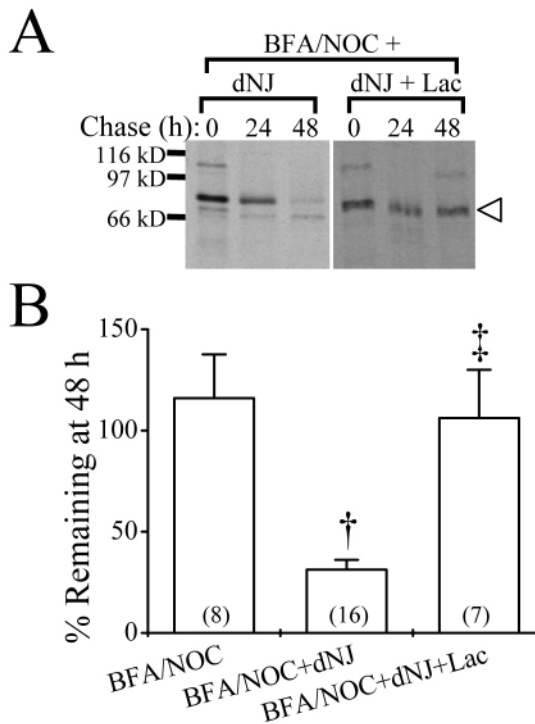
**Fig. 4.** ER retention does not destabilize wild-type Shaker protein. (A) Shaker-expressing cells were treated with brefeldin A (5  $\mu\text{g/ml}$ ) and nocodazole (20  $\mu\text{g/ml}$ ) (BFA/NOC) or were untreated. Cells were metabolically labeled for 10 minutes and were harvested immediately (0 h) or after chase periods of 24 or 48 hours, as indicated. Open and closed arrowheads indicate the positions of the immature and mature forms of the Shaker protein, respectively. Compiled data from 5-8 similar experiments indicated that the amount of Shaker protein remaining at 48 hours was  $94\pm 12\%$  in untreated cells and  $116\pm 22\%$  in BFA/NOC-treated cells. (B,C) Representative differential interference contrast (DIC) (i) and confocal images (ii-iv) of HEK293T cells transiently transfected with wild-type Shaker are shown. At 24 hours post-transfection, cells were incubated in the presence of BFA/NOC (B) or left untreated (C), before permeabilization, labeling with antibodies against calnexin (ii) and Shaker (iii), and visualization with fluorescently conjugated secondary antibodies. Red, calnexin; green, Shaker. The merged images are shown in iv. Bars, 10  $\mu\text{m}$ .

competent for ER to Golgi transport, these data strongly support the conclusion that the protein folds and assembles properly in cells treated with BFA/NOC and dNJ.

To further support this conclusion, we repeated the experiment with a misfolded Shaker mutant protein, D316K. We have previously shown that D316K in transmembrane segment S3 prevents proper folding by disrupting an electrostatic structural interaction with a conserved S4 residue, K374 (Tiwari-Woodruff et al., 1997). In contrast to the wild-type protein, D316K failed to mature to a complex glycosylated form on removal of BFA/NOC in the continued presence of dNJ (Fig. 6C). Similar results were also obtained with another misfolded Shaker mutant, K374E in S4 (data not shown). Thus, the behavior of the wild-type protein on BFA/NOC removal is distinct from that of misfolded mutant proteins, consistent with the conclusion that wild-type Shaker folds and assembles properly when it is trapped in the ER and prevented from interacting with calnexin.

#### Transient interaction with calnexin provides long-term protection from ERAD

Our data indicate that association with calnexin can protect the properly folded wild-type Shaker protein from degradation when it is in the ER. However, our previously published results establish that the interaction between Shaker and calnexin is transient, peaking shortly after synthesis of the Shaker protein and declining quickly thereafter (Nagaya et al., 1999). This rapid time course, which was measured in the absence of BFA/NOC treatment, is quite different from that of the relatively slow proteasomal degradation of N259Q+N263Q (Khanna et al., 2001) and of the wild-type protein expressed in cells treated with BFA/NOC and dNJ (Fig. 5). Therefore, we investigated whether prolonged interaction with calnexin is required for protection from degradation in the ER. In particular, we determined whether BFA/NOC treatment altered the time course of interaction between the wild-type Shaker protein and calnexin (Fig. 7). The Shaker protein was expressed in cells treated in the presence or absence of BFA/NOC and metabolically labeled during a 2 minute pulse. Association with calnexin was assessed immediately after the pulse and at various times of chase. The time course of calnexin

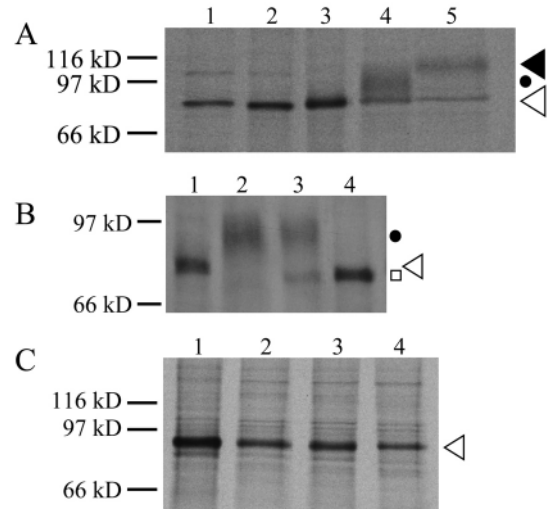


**Fig. 5.** Wild-type Shaker is destabilized when calnexin interaction and ER exit are blocked. (A) Shaker-expressing cells were treated with BFA/NOC and dNJ (1 mM) added during the starvation, pulse and chase periods; or with BFA/NOC, dNJ and Lac (10  $\mu$ M). Cells were labeled for 10 minutes and chased for 0, 24 or 48 hours as indicated. Open arrowhead indicates the position of the core-glycosylated, immature form of the Shaker protein. (B) The amount of core glycosylated Shaker protein remaining at 48 hours of chase was quantified by densitometry and expressed relative to the amount present at 0 time of chase (100%). The number of experiments for each condition (n) is shown in parentheses on the appropriate bar.  $^{\dagger}P < 0.0001$  compared with BFA/NOC;  $^{\ddagger}P < 0.0001$ , compared with BFA/NOC+dNJ (Student's *t*-test).

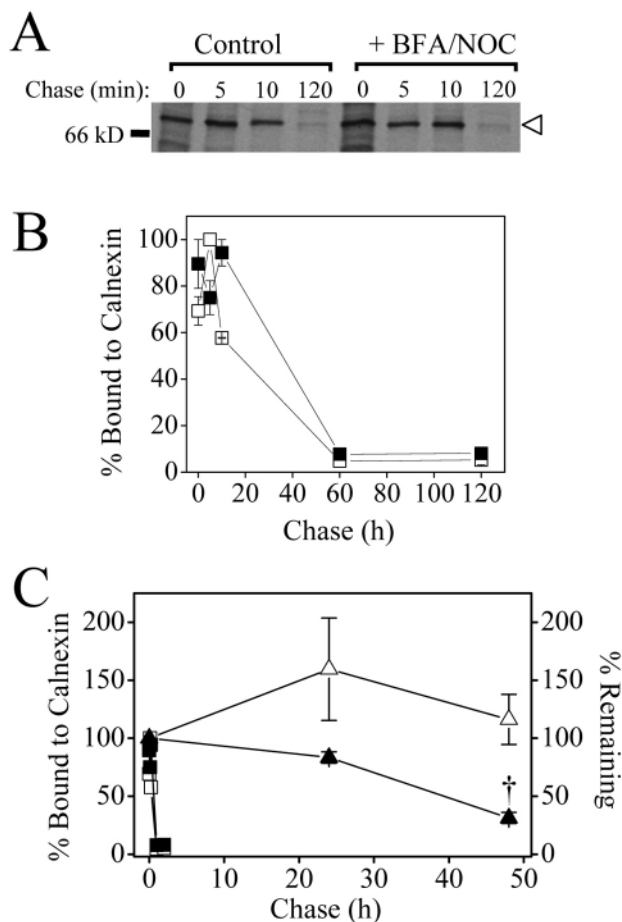
interaction was similar with or without treatment with BFA/NOC (Fig. 7A,B). Dissociation of wild-type Shaker from calnexin occurred much faster than the time course of degradation seen when calnexin association was prevented with dNJ (Fig. 7C). These results indicate that prolonged interaction with calnexin is not the mechanism by which ER-trapped Shaker protein is protected from proteasomal degradation. Therefore, transient interaction with calnexin confers long-lasting protection from degradation on properly folded Shaker protein localized in the ER. It is worth noting that we were unable to detect any interaction between the wild-type Shaker protein and calreticulin (data not shown). Thus, it is unlikely that interaction with calreticulin protects the Shaker protein from degradation.

#### ER mannosidase I inhibitor prevents ERAD of the wild-type Shaker protein localized in the ER

For some ERAD substrates, mannose trimming by ER mannosidase I is required for proteasomal degradation (Jakob et al., 1998; Chillaron et al., 2000; Chung et al., 2000; Wang



**Fig. 6.** Shaker wild-type protein remains competent for ER-to-Golgi transport despite treatment with BFA/NOC and dNJ. (A) Cells expressing the Shaker wild-type protein were preincubated with BFA/NOC and dNJ for 2 hours; the drugs were then maintained during a 30 minute starvation period, 30 minute pulse and 2 hour chase. After 2 hours of chase, cells were washed with PBS and the medium was replaced. The chase was then continued in the presence of dNJ with or without BFA/NOC. At various times, cells were harvested and solubilized with detergent, and the Shaker protein was immunoprecipitated and subjected to electrophoresis and fluorography. Lanes show Shaker protein from cells: (1) harvested immediately after pulse; (2) harvested after 2 hours of chase; (3) harvested after 9 hours of chase in the presence of dNJ and BFA/NOC; (4) harvested after 9 hours of chase in the presence of dNJ but no BFA/NOC after the first 2 hours of chase; (5) untreated with dNJ and BFA/NOC and harvested after 9 hours of chase. Open arrowhead indicates the position of the immature form of the Shaker protein. Closed arrowhead indicates the position of the mature form of the Shaker protein from untreated cells (lane 5). Filled circle indicates the position of a diffuse band seen upon removal of BFA/NOC (lane 4). This putative mature band was first detected approximately 2-3 hours after the removal of BFA/NOC. Representative results are shown from a total of six experiments. (B) Cells expressing wild-type Shaker were preincubated, starved, pulsed and chased for 2 hours in the presence of dNJ and BFA/NOC. At 2 hours of chase, cells were washed with PBS and the medium was replaced. The chase was then continued for 3 more hours in the presence of dNJ with (lane 1) or without (lanes 2-4) BFA/NOC. Lane 3: immunoprecipitated protein was treated with endo H. Lane 4: immunoprecipitated protein was treated with PNGase F. Filled circle indicates the position of the diffuse band seen upon removal of BFA/NOC. Open arrowhead indicates the position of the immature form of the Shaker protein. Open square indicates the deglycosylated Shaker protein. Representative results are shown from a total of two experiments. (C) Cells expressing the misfolded Shaker mutant D316K were preincubated, starved, pulsed and chased for 2 hours in the presence of dNJ and BFA/NOC. The chase was then continued in the presence of dNJ with or without BFA/NOC. Lanes show D316K protein from cells: (1) harvested after 2 hours of chase; (2) harvested after 6 hours of chase in presence of dNJ and BFA/NOC; (3) harvested after 6 hours of chase in presence of dNJ but no BFA/NOC after the first 2 hours of chase; (4) untreated with dNJ and BFA/NOC and harvested after 6 hours of chase. Open arrowhead indicates the position of the immature form of the Shaker protein. Representative results are shown from a total of two experiments.



**Fig. 7.** Transient interaction with calnexin provides long-term protection from ERAD. (A) Shaker-expressing cells were treated with BFA/NOC or were untreated, as indicated. Cells were metabolically labeled for 2 minutes, chased for the indicated times and lysed. Proteins were precipitated sequentially with antibodies directed against calnexin and Shaker (Nagaya et al., 1999). A representative experiment is shown;  $n=3$ . Open arrowhead indicates the position of the core-glycosylated, immature form of the Shaker protein. (B) The amount of Shaker protein obtained after sequential immunoprecipitation with calnexin and Shaker antibodies in the presence (■) or absence (□) of BFA/NOC treatment was quantified by densitometry, normalized to the maximum value obtained during the experiment and plotted versus chase time. A representative experiment is shown;  $n=3$ . (C) Comparison of the time course of calnexin interaction and ERAD for the wild-type Shaker protein. The percentage of Shaker protein remaining at 0, 24 and 48 hours of chase after treatment with BFA/NOC (△) or with BFA/NOC and dNJ (▲) was quantified by densitometry and plotted versus time of chase. The values of the percentage of Shaker protein remaining in BFA/NOC-treated samples were  $160\pm44\%$  (24 hours) and  $83\pm4.9\%$  (48 hours) and in BFA/NOC+dNJ-treated samples were  $83\pm8\%$  (24 hours) and  $31\pm5\%$  (48 hours).  $^{\dagger}P<0.001$  for BFA/NOC/dNJ versus BFA/NOC at the 48 hour chase time; Student's *t*-test. The data from B have been replotted on the same axes using the same symbols as in B (□, ■).

and White, 2000; Fagioli and Sitia, 2001). Inhibition of ER mannosidase I by deoxymannojirimycin (dMJ), a general mannosidase inhibitor, or by kifunensine, a specific inhibitor of mannosidase I (Elbein et al., 1990), delays or prevents

ERAD. We therefore investigated the role of mannose trimming in the proteasomal degradation of the Shaker wild-type protein. We trapped wild-type Shaker in the ER with BFA/NOC, prevented interaction with calnexin by co- and post-translational treatment with dNJ, and determined the effect of dMJ on protein stability at 48 hours of chase (Fig. 8). Treatment with dMJ abolished the degradation of the Shaker protein under these conditions. Treatment with kifunensine also blocked ERAD of the Shaker protein. By contrast, treatment with swainsonine, a specific inhibitor of ER mannosidase II, did not prevent Shaker degradation. Thus, mannose trimming by ER mannosidase I helps to specify the fate of the glycosylated Shaker protein.

## Discussion

### Native channel proteins can be ERAD substrates

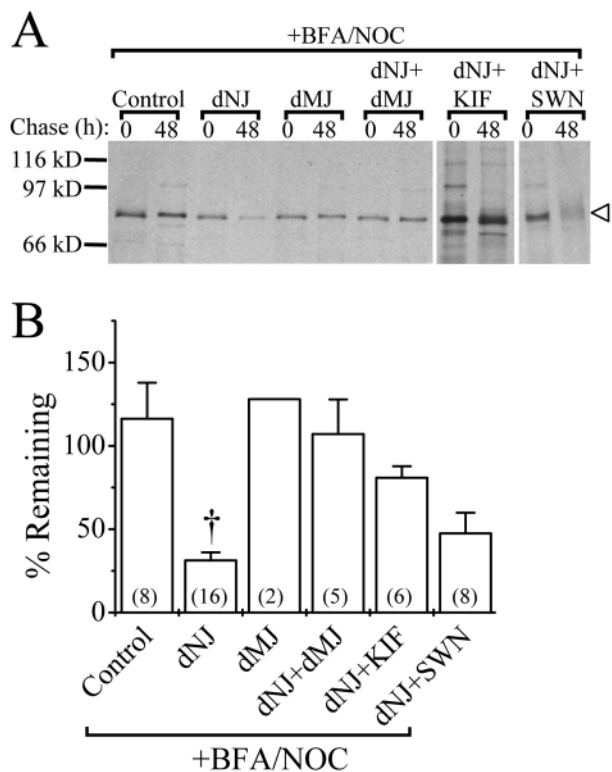
In this study, we have identified conditions that lead to proteasomal degradation of the wild-type Shaker protein despite apparently proper folding and assembly. This is not the first example of a native ERAD substrate. HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, is an ER-resident protein that is degraded by proteasomes in a sterol-regulated manner to maintain cholesterol homeostasis (Hampton, 2002). Among proteins destined for post-ER locations, however, ERAD generally functions to rid the cell of misfolded or damaged molecules (Kopito, 1997). The structural defects of ERAD substrates may be subtle. For instance, a single amino acid deletion,  $\Delta F508$ , in the cystic fibrosis transmembrane conductance regulator (CFTR), is the most common mutation contributing to cystic fibrosis. Although several lines of evidence indicate that the  $\Delta F508$  mutation results in a temperature-sensitive folding defect, the protein retains residual functional activity (Denning et al., 1992). Thus, ERAD substrates can include native and near-native proteins that would be functional if they were transported to their appropriate destinations. In accord with this conclusion, a recent study has shown that UDP-glucose:glycoprotein glucosyltransferase, the folding sensor of the calnexin/calreticulin chaperone system, can recognize subtle structural changes in mutant proteins that retain full enzymatic activity (Taylor et al., 2004).

### Calnexin interaction protects wild-type Shaker protein from ERAD targeting machinery

The wild-type Shaker protein is degraded by cytoplasmic proteasomes when it is trapped in the ER and prevented from interacting with the lectin chaperone, calnexin. Neither condition alone is sufficient to destabilize the protein. These results can account for the instability of the unglycosylated N259Q+N263Q Shaker mutant in mammalian cells (Khanna et al., 2001). Proteasomal degradation of the wild-type protein is abolished when mannosidase I trimming of the core glycan is inhibited.

Our results are consistent with the conclusion that the wild-type Shaker protein folds and assembles properly when it is trapped in the ER and prevented from interacting with calnexin. On removal of BFA/NOC, the wild-type protein exited the ER to acquire complex carbohydrates in the Golgi apparatus. The resulting mature band was similar but not





**Fig. 8.** ER mannosidase I inhibitors block ERAD of wild-type Shaker protein. (A) Shaker-expressing cells were pulsed for 10 minutes and either harvested immediately or chased for 48 hours. Cells were treated with BFA/NOC and no additional drug; BFA/NOC and dNJ (1 mM); BFA/NOC and dMJ (1 mM); BFA/NOC, dNJ and dMJ; BFA/NOC, dNJ and KIF (10  $\mu$ M); or BFA/NOC, dNJ and SWN (1 mM). A representative fluorograph is shown. Open arrowhead indicates the position of the core glycosylated, immature form of the Shaker protein. (B) The amount of core glycosylated Shaker protein remaining at 48 hours of chase was quantified by densitometry and expressed relative to the amount present at 0 time of chase (100%). The number of experiments for each condition (n) is shown in parentheses on the appropriate bar.  $^{\dagger}P < 0.001$  compared with all other treatments except dNJ+SWN; Student's *t*-test. Data are presented as mean  $\pm$  s.e.m. except for the dMJ sample, where  $n = 2$ . For the dMJ sample, the mean value is shown.

identical to that obtained in untreated cells. The differences in electrophoretic mobility and appearance may be due to prolonged exposure of the core glycan to ER-processing enzymes during BFA/NOC treatment, with consequences for the final structure of the complex carbohydrate generated in the Golgi compartment. In contrast to the wild-type protein, misfolded Shaker mutants did not exit the ER on removal of BFA/NOC. Thus, our data lend strong support to the hypothesis, first suggested on the basis of work with the unglycosylated Shaker mutant protein, that properly folded and assembled  $K^+$  channel proteins can be targeted for ERAD. However, we cannot completely rule out the possibility that folding of the wild-type protein is subtly altered in cells treated with BFA/NOC and dNJ.

Our results indicate that brief association with calnexin confers long-lasting protection from ERAD on the wild-type Shaker protein when it is localized in the ER. After interacting

transiently with calnexin, Shaker wild-type is stable in the ER for up to 48 hours. By contrast, if association with calnexin is prevented by co-translational treatment with dNJ, ER-trapped Shaker protein is substantially degraded over a 48 hour period. Calnexin interaction has been shown to inhibit degradation of other ERAD substrates, and in some cases, evidence of prolonged interaction with the chaperone has been presented (Wang and White, 2000). By contrast, it is clear that interaction with calnexin protects the Shaker protein long after it has dissociated from the chaperone.

It remains to be determined how transient interaction with calnexin confers its long-lasting protection on the Shaker protein. One possibility is that calnexin interaction helps to deliver the protein to ER exit sites where it is sequestered from the dislocation machinery that targets ERAD substrates to cytoplasmic proteasomes (Barlowe, 2000). This idea is consistent with the observation that ER exit, estimated by the rate of maturation, is slowed when calnexin interaction is prevented.

Although calnexin interaction accelerates ER exit of the wild-type protein, it is clear that acquisition of ER-to-Golgi transport competence is a multistep process. We have previously shown that misfolded and unassembled mutant forms of the Shaker protein interact transiently with calnexin with a time course similar to that of the wild-type protein (Nagaya et al., 1999). Thus, mutant subunits with defects in subunit assembly, voltage sensor folding and pore formation escape the folding sensor of the calnexin chaperone system (Nagaya et al., 1999), UDP-glucose:glycoprotein glucosyltransferase (Helenius and Aebi, 2001). These mutant proteins fail to become competent for ER exit, are retained in the ER and are subjected to ER-associated disposal (Schulzeis et al., 1998) (M. P. Myers, R. Khanna, E. J. Lee and D. M. Papazian, unpublished). Therefore, at least one additional folding sensor downstream of the calnexin system helps to assess the structural integrity of Shaker proteins and sorts them for ER retention or forward trafficking. It is likely that additional chaperones participate in these downstream quality control checkpoints.

#### Core glycan may promote forward trafficking independent of calnexin interaction

Our results raise the possibility that the core glycan serves as a positive determinant for forward trafficking of the properly folded Shaker protein independently of calnexin interaction. This idea arises from comparing the fate of N259Q+N263Q with that of the wild-type protein treated co-translationally with dNJ but in the absence of BFA/NOC so that forward trafficking is not precluded. When calnexin interaction is prevented pharmacologically, the wild-type protein leaves the ER and matures efficiently, albeit at a somewhat reduced rate. By contrast, when calnexin interaction is prevented by eliminating *N*-linked glycosylation, the N259Q+N263Q protein fails to exit the ER efficiently and is degraded by proteasomes (Khanna et al., 2001). These results suggest that, in the absence of calnexin interaction, there is a kinetic competition between forward trafficking and ERAD. For the wild-type protein treated co-translationally with dNJ, forward trafficking is fast compared with degradation. Furthermore, the wild-type protein is stable, presumably because it is separated

from the ERAD targeting apparatus on leaving the ER. By contrast, for the unglycosylated N259Q+N263Q protein, targeting to proteasomes occurs before ER exit (Khanna et al., 2001). Assuming that N259Q+N263Q achieves the native structure as efficiently as the wild-type protein, as suggested by the C96/C505 disulfide bond assay (Khanna et al., 2001), then the outcome of the competition between forward trafficking and ERAD depends on the presence or absence of *N*-linked oligosaccharides.

How might the core glycan promote forward trafficking? One possibility is suggested by the observation that proteasomal degradation of the ER-trapped wild-type protein treated co-translationally with dNJ is abolished when trimming by ER mannosidase I is inhibited. One possibility is that mannose trimming is required for proteasomal targeting of the Shaker protein, as has been found for other ERAD substrates (Liu et al., 1997; Roth et al., 2002). However, we have shown that N259Q+N263Q is targeted to the proteasome with similar kinetics despite the complete absence of oligosaccharide modification (Khanna et al., 2001). This raises the possibility that the man<sub>8,9</sub>-containing core glycan, which has not been trimmed by ER mannosidase I, may act as a positive determinant for ER-to-Golgi transport. Alternatively, the glycosylated and unglycosylated forms of the Shaker protein may be targeted for ERAD by glycan-dependent and glycan-independent pathways, respectively. Finally, we cannot rule out the possibility that the unglycosylated N259Q+N263Q protein contains a subtle structural defect not detected by the C96/C505 disulfide assay, leading to recognition and retention by the quality control machinery. Further experiments will be necessary to distinguish between these possibilities.

#### Quality control of K<sup>+</sup> channel biogenesis

Our results suggest the following picture of quality control during channel biogenesis. Normal quality control of K<sup>+</sup> channel biogenesis involves a minimum of two folding checkpoints. All quality control checkpoints must be passed for the protein to exit the ER. Calnexin interaction early in biogenesis promotes forward trafficking and confers protection from ERAD for properly folded Shaker protein. In the absence of calnexin interaction, there is a kinetic competition between forward trafficking and ERAD. The core glycan, in particular the mannosidase I-untrimmed glycan, may serve as a positive determinant for ER-to-Golgi trafficking. Although K<sup>+</sup> channel quality control may be more complicated, the proposed scheme can account for all of the data that we have obtained with the wild-type and N259Q+N263Q proteins, as well as misfolded and unassembled mutants.

Our scheme is compatible with a recent study which found that unglycosylated Shaker protein trafficked out of the ER significantly more slowly than the glycosylated protein (de Souza and Simon, 2002). Somewhat paradoxically, the cell-surface expression of the glycosylated and unglycosylated proteins did not differ significantly in that study. This result contrasts with our previously reported finding that glycosylation dramatically increases the surface expression of the Shaker protein (Khanna et al., 2001). One possible explanation for this discrepancy is that different cell-surface labeling procedures were used. De Souza and Simon measured cell-surface expression by biotinylating endogenous lysine

residues, several of which are located in the S1-S2 loop quite close to the glycosylated asparagine residues (N259, N263). If these residues are biotinylated more efficiently in the absence of *N*-linked carbohydrate, the cell-surface expression of the unglycosylated protein will be overestimated. By contrast, we measured cell-surface expression by biotinylation of a single engineered cysteine residue (S346C) located outside of the glycosylated S1-S2 loop. De Souza and Simon also found that glycosylated and unglycosylated forms of the Shaker protein were degraded with similar kinetics. Again, this contrasts with our previously reported results. We found that N259Q+N263Q turned over with a half-time of approximately 18 hours, whereas the glycosylated wild-type protein was stable for at least 48 hours. This discrepancy is likely to be due to the fact that de Souza and Simon used epitope-tagged versions of N259Q+N263Q and wild-type Shaker. Both of these epitope-tagged proteins turned over with a half-time of approximately 6 hours, substantially faster than either the untagged N259Q+N263Q or wild-type proteins (Khanna et al., 2001). Of course, we cannot rule out the possibility that the use of different cell types accounts for some of the differences in the reported results. Apart from the technical issues regarding cell-surface biotinylation and the destabilizing effects of epitope tags; however, the main result of de Souza and Simon is that glycosylation dramatically accelerates ER exit of the Shaker protein, which is consistent with our findings.

Our results imply that calnexin plays an important role in the quality control of K<sup>+</sup> channel biogenesis, despite the fact that mutant subunits with defects in subunit assembly, voltage sensor folding and pore formation escape the folding sensor of the calnexin chaperone system (Nagaya et al., 1999). In addition to increasing the ER exit rate, it provides long-term protection from ERAD for properly folded K<sup>+</sup> channel proteins. This is not an issue in cultured HEK293T cells, where forward trafficking of the wild-type Shaker protein is extremely efficient. However, long-term protection from ERAD could be important for preserving properly folded K<sup>+</sup> channel subunits in their native cellular environments during regulated forward trafficking to control the density of cell-surface K<sup>+</sup> channels. For instance, during the activation of T lymphocytes, Kv1.3 channels are recruited to the cell surface from a pre-existing intracellular pool (Cai et al., 1992; Lewis and Cahalan, 1995). In myometrium, estrogen regulates the cell-surface trafficking of Kv4.3 channels (Song et al., 2001). Thus, under a variety of physiological conditions, properly folded K<sup>+</sup> channel subunits may be retained intracellularly for extended periods of time.

Mutations in ion channel genes underlie a variety of human diseases (Lehmann-Horn and Jurkat-Rott, 1999; Shieh et al., 2000). In some of these 'channelopathies', channel proteins are retained in the ER and may be subjected to ERAD (Zhou et al., 1999; Ficker et al., 2000; Manganas et al., 2001; Rea et al., 2002). Investigating the mechanisms that lead to the ER retention and degradation of channel proteins will increase our understanding of the etiologies of these channelopathies.

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