Role of glycosylation in cell surface expression and stability of HERG potassium channels

Qiuming Gong, Corey L. Anderson, Craig T. January, and Zhengfeng Zhou. Role of glycosylation in cell surface expression and stability of HERG potassium channels. Am J Physiol Heart Circ Physiol 283: H77–H84, 2002. First published March 7, 2002; 10.1152/ajpheart.00008.2002.—The human ether-à-go-go-related gene (HERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel in the heart. We previously showed that HERG channel protein is modified by N-linked glycosylation. HERG protein sequence contains two extracellular consensus sites for N-linked glycosylation (N598, N629). In this study, we used the approaches of site-directed mutagenesis and biochemical modification to inhibit N-linked glycosylation and studied the role of glycosylation in the cell surface expression and turnover of HERG channels. Our results show that N598 is the only site for N-linked glycosylation and that glycosylation is not required for the cell surface expression of functional HERG channels. In contrast, N629 is not used for glycosylation, but mutation of this site (N629Q) causes a protein trafficking defect, which results in its intracellular retention. Pulse-chase experiments show that the turnover rate of nonglycosylated HERG channel is faster than that of the glycosylated form, suggesting that N-linked glycosylation plays an important role in HERG channel stability.

Although electrophysiological and pharmacological properties of HERG channels have been studied widely, less is known about the biochemical processing of HERG channel protein. We previously reported (37, 38) that wild-type HERG channel protein expressed in HEK293 cells exhibits two bands on Western blot analysis and that the generation of both bands involves asparagine (N)-linked glycosylation. We showed (37) that the larger-molecular-mass band is the fully glycosylated, mature form of HERG channel located in the plasma membrane and the smaller-molecular-mass band is a core-glycosylated, precursor form of HERG channel located in the endoplasmic reticulum (ER). The HERG protein sequence contains two extracellular consensus sites for N-linked glycosylation (N598, N629). Recently, Petrecca et al. (20) reported that both single (N598Q, N629Q) and double (N598Q-N629Q) mutations resulted in the intracellular retention of HERG channel protein with no HERG K⁺ current detected. Those authors suggested that both consensus sites are targets for N-linked glycosylation and that N-linked glycosylation is required for the cell surface expression of HERG channels. In the present experiments we provide evidence that, of the two N-linked glycosylation consensus sites, only N598 undergoes glycosylation. We also show that glycosylation at N598 is not obligatory for the assembly of functional channels and their transport to the cell surface. In contrast, the N629 site is not involved in the glycosylation process; rather, mutation of the N629 site (N629Q) causes a protein trafficking defect that results in the intracellular retention of the mutant channel protein. Finally, our results show that the turnover rate of nonglycosylated HERG channel is faster than that of the glycosylated form, suggesting that N-linked glycosylation plays an important role in HERG channel stability.

MATERIALS AND METHODS

DNA constructs and transfection of HEK293 cells. The wild-type cDNA encoding HERG was obtained from Dr. Gail Robertson (University of Wisconsin; Ref. 34). The HERG mutations were generated by site-directed mutagenesis with

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the GeneEditor in vitro mutagenesis system (Promega). Each mutation was verified by DNA sequencing. Wild-type and mutant cDNAs were subcloned into BamHI and EcoRI sites of the pcDNA3 vector (Invitrogen). For green fluorescent protein (GFP)-tagged HERG (HERG-GFP) constructs, the stop codon of HERG was removed and replaced by an EcoRI site with the GeneEditor in vitro mutagenesis system. The constructs were then subcloned in-frame into HindIII and EcoRI sites of pEGFP-N2 vector (Clontech). Thus GFP is attached to the COOH terminus of HERG with EcoRI sites of pEGFP-N2 vector (Clontech). Thus GFP is transiently or stably transfected with these constructs by using a lipofectamine method as previously described (37). When transiently transfected cells were used for patch-clamp experiments, GFP cDNA (1 µg) was cotransfected with untagged HERG cDNA (5 µg) to serve as an indicator.

**Western blot analysis.** Membrane protein preparation and Western blot procedures were previously described (38). The membrane proteins were subjected to SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. The nitrocellulose membranes were incubated with an anti-HERG antibody raised against the HERG-thioredoxin fusion protein (37). The antibody was detected with an ECL detection kit (Amersham).

For experiments with endoglycosidase H (Endo H) treatment, 30 µg of cell membrane protein was dissolved in 30 µl of 50 mM sodium citrate buffer (pH 5.5) containing 15 mM β-mercaptoethanol and 0.12% SDS by boiling for 2 min. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM followed by addition of 15 µl of Endo H. The mixture was incubated at 37°C for 24 h. The reaction was stopped by adding sample buffer and boiling for 2 min.

**Labeling of cell surface proteins with biotinylation reagent.** Cell surface proteins were labeled with a membrane-impermeant biotinylation reagent, sulfo-NHS-biotin (sulfo-NHS-SS-biotin; Ref. 3). HEK293 cells stably expressing wild-type HERG, N598Q, and N629Q were washed twice with ice-cold PBS and labeled with 3 ml of 1 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS (pH 8.0) for 30 min at 4°C to limit endocytosis of labeling reagent. After two washes with ice-cold PBS, the cells were incubated in PBS containing 100 mM glycine to quench the unreacted biotin reagent for 20 min at 4°C and washed again with ice-cold PBS. Cells were then lysed with 500 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mg/ml BSA) with a protease inhibitor cocktail. After centrifugation at 14,000 rpm for 5 min at 4°C, the cell lysate was preclearred by incubation with protein A-agarose beads (Pierce). HERG antiserum (1:100 dilution) was then added, and the mixture was incubated at 4°C overnight. The antigen-antibody complexes were isolated with fresh protein A-agarose beads that had been blocked by cell lysate from nontransfected cells to minimize nonspecific binding. The immunoprecipitates were washed four times with the immunoprecipitation buffer. The bound antigen was eluted from the protein A-agarose beads by sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. After being transferred onto nitrocellulose membranes, the biotin-labeled HERG proteins were detected by using horseradish peroxidase (HRP)-conjugated streptavidin (Pierce) and an enhanced chemiluminescence detection kit (Amersham).

**Pulse-chase metabolic labeling and immunoprecipitation.** Pulse-chase metabolic labeling and immunoprecipitation were performed as previously described (37). Briefly, wild-type or mutant HERG-transfected cells were starved for 1 h in serum-free DMEM lacking methionine and cysteine and containing 0.25% BSA. Cells were then incubated in the same medium containing [35S]methionine/cysteine (400 µCi/ml). After 1 h of labeling, the medium was removed and cells were chased in DMEM with 2 mM unlabeled methionine and cysteine. At different time intervals, the cells were lysed in 500 µl of the immunoprecipitation buffer. HERG proteins were immunoprecipitated with anti-HERG antibody. The precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis and visualized with autoradiography.

**Inhibition of N-linked glycosylation by tunicamycin.** For Western blot and patch-clamp experiments, HEK293 cells were transiently transfected with wild-type HERG-pcDNA3 construct, and 10 µg/ml of tunicamycin was included in the culture medium continuously before and after transfection. The cells were used for Western blot and patch-clamp experiments 48 h after transfection. For pulse-chase experiments, the cells stably expressing wild-type HERG were metabolically labeled and chased as described above and 10 µg/ml of tunicamycin was included in the culture medium continuously during starvation, labeling, and chase procedures.

**Patch-clamp recordings.** Membrane currents were recorded in whole cell configuration with suction pipettes as previously described (38). Cells were superfused with HEPES-buffered Tyrode solution containing (in mM) 137 NaCl, 4 KCl, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The internal pipette solution was composed of (in mM) 130 KCl, 1 MgCl2, 5 EGTA, 5 MgATP, and 10 HEPES (pH 7.2 with KOH). An Axopatch-1D patch-clamp amplifier was used to record membrane currents. Computer software (pCLAMP) was used to generate voltage-clamp protocols and to acquire and analyze current signals. All experiments were performed at 22–23°C. Data are presented as means ± SE. Student’s t-test was used for statistical analysis.

**RESULTS**

Figure 1 shows the proposed model of membrane topology for the HERG channel protein. Similar to other voltage-gated K+ channels, HERG has six transmembrane domains (S1–S6) with the cytoplasmic NH2-terminal and COOH-terminal regions, and the coassembly of four of these protein subunits is thought to form a functional ion channel. The HERG protein sequence contains two N-linked glycosylation consensus sites (N-X-T/S), where X is any amino acid except proline, in the S5–S6 extracellular linker (35). One site (N598) is located 26 amino acids downstream of the S5 transmembrane domain, and the other (N629) is located within the pore region and is 9 amino acids upstream of the S6 transmembrane domain. To identify the site(s) for N-linked glycosylation of HERG protein, we mutated asparagine (N) to glutamine (Q) at these two consensus sites, N598Q and N629Q. We expressed these two mutants in HEK293 cells by stable transfections and analyzed HERG channel protein by Western blot with a specific HERG antibody (Fig. 2). Wild-type HERG expressed two protein bands, a 155 (upper) and a 135 (lower)-kDa band. We previously showed (37) that the 155-kDa band is the fully glycosylated, mature form of HERG channel protein located in the plasma membrane and the 135 kDa band is a core-glycosylated, precursor form of HERG channel protein located in the ER. On Western blot, the N598Q mutation expressed only one band with a molecular...
mass of 132 kDa, which is slightly smaller than that of the 135-kDa band of wild-type HERG. The N629Q mutation also expressed a single band but with a molecular mass of 135 kDa, which is similar to the molecular mass of the lower band of wild-type HERG, suggesting that it is a core-glycosylated form of HERG channel protein. To study further the glycosylation of wild-type and mutant HERG channels, we used Endo H to digest high-mannose oligosaccharides that are added during core glycosylation of newly synthesized proteins in the ER. As we showed previously (37), the 155-kDa band of the wild-type HERG protein was resistant to Endo H digestion, whereas the 135-kDa band of wild-type HERG was sensitive to Endo H treatment. For the N598Q mutation, the single 132-kDa band was resistant to Endo H digestion, suggesting that N598Q is not glycosylated. For the N629Q mutation, the single 135-kDa band was sensitive to Endo H, with its molecular mass reduced to 132 kDa. These results suggest that N629Q mutant protein undergoes core glycosylation in the ER but then fails to be transported to the medial Golgi to undergo complex glycosylation.

Using GFP-tagged HERG constructs, Petrecca et al. (20) showed that N-linked glycosylation is required for the cell surface expression of functional HERG channels. To study the cell surface expression of wild-type HERG, N598Q, and N629Q, we performed cell surface labeling of membrane proteins with a membrane-impermeant biotinylating reagent, sulfo-NHS-SS-biotin. Biotinylated HERG channels were then immunoprecipitated by HERG antibody and detected by streptavidin-HRP (Fig. 3). Wild-type HERG was labeled by the biotin reagent and showed a band with molecular mass of ~155 kDa, which corresponds to the mature form of HERG channel protein found in Western blot experiments. The N598Q mutant was also labeled by the biotin reagent and showed a band at ~132 kDa, suggesting that N598Q is expressed in the cell surface membrane. In contrast, N629Q was not labeled by the biotin reagent. These results, together with the results...

Fig. 1. Proposed transmembrane topology of the human ether-a-go-go-related gene (HERG) K⁺ channel. The amino acid sequence between S5 and S6 is shown, and the N-linked glycosylation consensus sequences involving N598 and N629 are underlined.
of the Endo H experiments, suggest that N629Q is not expressed in the cell surface membrane and is retained in the ER. As control experiments, no detectable band was observed in nontransfected HEK293 cells or in wild-type HERG-transfected cells without labeling with biotinylating reagent.

The above biotin cell surface labeling experiments indicate that N598Q is expressed in the cell surface membrane, whereas N629Q is not. To test whether these two mutants express functional channels, we performed patch-clamp recordings of HERG currents from cells stably transfected with wild-type HERG and these mutant channels (Fig. 4). The wild-type HERG current exhibited voltage-dependent activation with inward rectification at more positive voltages. The inward rectification was analyzed with normalized tail current amplitude measured after the repolarizing step to −50 mV. The cells were then clamped to −50 mV for 6 s to record a tail current.

\[ |V| = \frac{\text{normalized tail current}}{\text{wild-type HERG}} \]

The half-maximal activation voltages for the wild-type and N598Q were 7.00 ± 0.22 and 7.03 ± 0.30 (P > 0.05) mV, respectively. These results indicate that N-linked glycosylation at position N598 did not alter the voltage dependence of HERG channel activation. In contrast, the N629Q mutation failed to generate HERG current. This finding is in agreement with the biotin labeling and Endo H experiments and suggests that the N629Q mutation causes a protein trafficking defect that results in failure of the core-glycosylated mutant channel to reach the cell surface membrane. To determine whether the protein trafficking defect is due to disruption of N-linked glycosylation at N629, we studied another mutant, S631A, which also disrupts the consensus site (N-X-T/S) involving N629. Western blot analysis showed that S631A expressed both the core-glycosylated (135 kDa) and fully glycosylated (155 kDa) forms of HERG protein similar to those of wild-type HERG (Fig. 5). Patch-clamp recordings showed that S631A generated HERG current with reduced inactivation (Fig. 4; Ref. 11). We also studied a mutant construct with double mutations N598Q-S631A, which disrupts both consensus sites. The N598Q-S631A expressed only one band with the same molecular mass as that of N598Q (132 kDa; Fig. 5). The current generated by N598Q-S631A also showed reduced inactivation similar to that observed in S631A (Fig. 4). The fact that disruption of N629 glycosylation site by mutation of S631A did not change the glycosylation pattern of HERG channel protein suggests that the consensus site N629 is not used for N-linked glycosylation. Thus our results demonstrate that N598 is the only site for N-linked glycosylation in HERG channel protein and that glycosylation at this site is not required for the assembly of functional channels or for their transport to the cell surface.

Our results are different from those reported by Petrecca et al. (20), who suggested that both the N598 and N629 sites are glycosylated and that glycosylation is required for the cell surface expression of HERG channels. In their experiments, they used GFP-tagged HERG constructs. To determine whether the differences are due to the attachment of GFP to HERG, we also made GFP-tagged HERG (HERG-GFP) constructs and expressed GFP-tagged HERG channels in HEK293 cells.
cells by transient transfection. The results showed that, similar to untagged HERG channels, wild-type HERG-GFP and N598Q-GFP generated HERG currents and N629Q-GFP failed to generate HERG current (Fig. 6). These results suggest that under our experimental conditions GFP tagging did not alter the functional expression of wild-type HERG and mutant channels.

To further investigate the role of N-linked glycosylation in the cell surface membrane expression and function of HERG channels, we also used tunicamycin to inhibit N-linked glycosylation. Figure 7 shows the effects of tunicamycin on HERG protein and current of wild-type channels transiently expressed in HEK293 cells. Western blot analysis showed that in the absence of tunicamycin both core-glycosylated and fully glycosylated forms of HERG protein were present. In the presence of tunicamycin, the Western blot showed one band with molecular mass of 132 kDa; similar to findings with the N598Q mutation, suggesting that tunicamycin treatment prevents N-linked glycosylation of HERG channel protein (Fig. 7A). Patch-clamp recordings showed that tunicamycin did not prevent expression of functional channel in the cell surface membrane (Fig. 7B). These results provide further support for the conclusion that N-linked glycosylation is not required for the assembly of functional channels or for their transport to the cell surface membrane.

To study further what role N-linked glycosylation may have in HERG channels, we performed pulse-chase experiments to evaluate the rate of HERG protein turnover (Fig. 8). In these experiments, HEK293 cells stably transfected with wild-type HERG or N598Q mutation were labeled for 1 h with [35S]methionine/cysteine and chased with unlabeled methionine and cysteine for different intervals between 4 and 24 h. For wild-type HERG channels, HERG protein was initially synthesized as a single band with a molecular mass of 135 kDa and was then gradually converted to a larger-molecular-mass band of 155 kDa. For N598Q mutation, HERG protein was initially synthesized as a single band with a molecular mass of ~132 kDa. It was not converted to a larger-molecular-mass protein because of the lack of N-linked glycosylation. However, the protein underwent progressive degradation, with complete disappearance of the band by 24 h. Similarly, in the presence of tunicamycin wild-type HERG also showed a faster turnover rate compared with the control. These findings suggest that HERG channel with-
out N-linked glycosylation is degraded more rapidly than glycosylated channel.

**DISCUSSION**

N-linked glycosylation is a common posttranslational modification of ion channel proteins. It is generally believed that N-linked glycosylation may control protein folding and intracellular trafficking, protect proteins from proteolytic degradation, and modify protein function (6, 10). However, the role of carbohydrate moieties varies among ion channel proteins. N-linked glycosylation has been reported to play an important role in the function of some ion channels. For example, the ROMK1 inward rectifier K⁺ channel shows reduced open probability when N-linked glycosylation is inhibited (29). For some voltage-gated ion channels, inhibition of N-linked glycosylation alters the voltage dependence of channel gating, such as Kv1.1 and Kv-LQT1/mink K⁺ channels (8, 32) and Na⁺ channels (36). N-linked glycosylation has also been reported to be required for the cell surface expression of ligand-gated channels including nicotinic acetylcholine receptor (16) and ATP purinergic receptors P2X1 and P2X2 (24, 33).

In contrast, other studies have shown that N-linked glycosylation is not required for the cell surface expression of cystic fibrosis transmembrane conductance regulator (17), cyclic nucleotide-gated channels (25), inward rectifier K⁺ channel GIRK1 (19), and voltage-gated K⁺ channels such as Kv1.1 and Shaker (5, 28).

Previous biochemical studies of HERG channels in transfected cells and native cardiac tissues have shown that HERG channel protein is modified by N-linked glycosylation (7, 20, 21, 37, 38). HERG protein sequence contains two extracellular consensus sites for N-linked glycosylation, N598 and N629. Recently, it was reported that N-linked glycosylation is required for the cell surface expression and that both glycosylation sites must be available for N-linked glycosylation to occur in HERG channel protein (20). In contrast, our experiments showed that inhibition of N-linked glycosylation by mutational disruption of consensus sites or by tunicamycin did not prevent the cell surface expression of functional HERG channels. The reason for this difference is not clear. In their experiments, Petrecca and colleagues (20) used GFP-tagged HERG constructs. However, our results with GFP-tagged HERG constructs demonstrated that the attachment of GFP to HERG channel protein had no effect on expression of functional wild-type HERG and N598Q mutant channels. The difference may result from the selection of stable cell lines. In fact, Petrecca et al. (20) reported that in one cell line stably expressing N598-GFP they were able to record a small HERG-like current. In our experiments we were able to record HERG current in multiple cell lines stably transfected with N598Q mutant, and the biotin surface labeling experiments also demonstrated the presence of N598Q channel protein in the cell surface membrane. From these findings, we conclude that the N-linked glycosylation at N598 is not required for the cell surface expression of functional HERG channels.

For the consensus site involving N629, both our results and those of Petrecca et al. (20) showed that replacement of the asparagine at position 629 with a glutamine resulted in the failure of N629Q mutant channel to traffic to the plasma membrane. Petrecca and colleagues (20) suggested that the trafficking defect of N629Q is due to the disruption of N-linked glycosylation at N629. However, our results indicate that the trafficking defect of N629Q is not caused by the disruption of N-linked glycosylation. In fact, N629 is not used for N-linked glycosylation. This conclusion is supported by the finding that the S631A mutant, which also disrupts the consensus site involving N629, exhibits a glycosylation pattern similar to that of wild-type HERG. Two reasons may account for the lack of N-linked glycosylation at the consensus site N629. First, it may be due to the presence of a proline at position 632. The presence of a proline at the position following the consensus sequence (N-X-T/S-P) has been reported to strongly reduce the likelihood of N-linked glycosylation (1, 9, 15, 26). Second, it has been reported that the efficiency of N-linked glycosylation at the N-X-T/S sequence depends on its distance from transmembrane segments. Efficient glycosylation sites are usually located >12 residues from the preceding transmembrane segment and >14 residues away from the following transmembrane segment (18, 22). The proposed topology model of HERG channel predicts that N629 is located at the ninth residue upstream from the S6 transmembrane domain of HERG channel. The observation that N629 is not a glycosylation site strongly suggests that the failure of the N629Q mutation to process to the surface membrane results from a trafficking defect due to the amino acid substitution rather than disruption of glycosylation. Thus it seems most likely that replacement of the asparagine with glutamine at position 629 may cause misfolding of HERG channel, leading to defective protein trafficking of the N629Q mutation.

The pulse-chase experiments indicate that N-linked glycosylation increases stability of HERG channel protein, whereas inhibition of N-linked glycosylation by mutation N598Q or by tunicamycin causes more rapid degradation of HERG channel protein. Decreased stability of nonglycosylated proteins has been reported for other ion channel proteins. Inhibition of N-linked glycosylation has been shown to reduce stability of acetylcholine receptor and Shaker K⁺ channel proteins (2, 12, 23). Although it is generally believed that N-linked glycosylation protects proteins from proteolytic degradation, the specific proteolytic system involved in the rapid degradation of nonglycosylated proteins is not fully understood. The accelerated degradation of nonglycosylated acetylcholine receptor has been shown to involve an intracellular lysosomal proteolytic system (23). Recently, it was reported that the rapid degradation of nonglycosylated Shaker K⁺ channel protein involves cytoplasmic proteasomes (12). Clearly, further studies are needed to elucidate the mechanism of the
rapid degradation of nonglycosylated HERG channel protein.

In summary, our experiments show that, of the two potential N-linked glycosylation sites N598 and N629 in HERG channel protein, only N598 is used for N-linked glycosylation. The glycosylation at this site is not required for the assembly of functional channels or for their transport to the cell surface. However, the nonglycosylated HERG channel shows an accelerated turnover rate, suggesting that N-linked glycosylation contributes to the protein stability of HERG channels.

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