Glycosylation of Eag1 (Kv10.1) Potassium Channels

INTRACELLULAR TRAFFICKING AND FUNCTIONAL CONSEQUENCES*

Eag1 is the prototypic member of the EAG family of voltage-gated potassium channels (1). Several studies have indicated a link between Eag1 and both proliferation and malignant transformation (2–4), but little is known about the physiological role of the channel in healthy tissues. Here we report on the impact of post-translational modifications upon the biological properties of the channel. N-Linked glycosylation is a common form of integral membrane protein processing that can profoundly affect protein expression, structure, and function. N-Linked oligosaccharides are assembled in a series of enzyme-catalyzed reactions. The reactions start with the addition of simple sugars in the ER1 lumen by co-translational transfer of preassembled core glycan to a nascent polypeptide. The oligosaccharides gain complexity as proteins progress through the Golgi apparatus, resulting in complex, high-mannose, or hybrid oligosaccharides bound to Asn-388 and 406. Asn-388 seems to undergo only core glycosylation, but complex sugars are bound to Asn-406. Correct complex glycosylation is required for proper trafficking of Eag1 to the plasma membrane but is also crucial for the correct function of channels already inserted in the membrane.

N-Linked glycosylation is a common post-translational modification of membrane proteins. Here we report that mature Eag1 potassium channels carry sugar moieties linked to asparagines at positions 388 and 406. Asn-388 seems to undergo only core glycosylation, but complex sugars are bound to Asn-406. Correct complex glycosylation is required for proper trafficking of Eag1 to the plasma membrane but is also crucial for the correct function of channels already inserted in the membrane.

The single most evident function of N-linked glycosylation is the promotion of proper folding of newly synthesized polypeptides in the ER (7, 8). When glycosylation is inhibited, the most commonly observed effect is the generation of misfolded, aggregated proteins that fail to reach a functional state and are subsequently degraded (7–11). Carbohydrates also stabilize the mature protein (12), partly by protecting it from degradation by proteases. A common effect is the generation of misfolded, aggregated proteins that fail to reach a functional state and are subsequently degraded (7–11). Carbohydrates also stabilize the mature protein (12), partly by protecting it from degradation by proteases. Glycosylation might also serve to enhance thermal stability (14) or influence the solubility of proteins as well as affect the intracellular transport and localization of the glycoprotein. Once expressed in fully mature form on the cell surface, N-linked glycans can influence functional properties like ligand binding or electrophysiological parameters (15). In the particular case of voltage-gated ion channels, glycosylation may also directly influence function through the charge of the sugar moiety. In this study, we have examined the glycosylation of Kv10.1 and its impact on the functional properties of the channel. We present evidence that proper complex glycosylation at one position, at the least, is crucial for both the proper transport and the functional properties of the channels. Our results highlight the concept that glycosylation must be considered as an important post-translational modification when studying ion channel function.

MATERIALS AND METHODS
DNA—All clones used were derived from a pTracer/hEAG1 construct (3). Mutations were generated with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s manual using the following primers: N286Q, 5’-GGTTGGCGGCTCCAGGATCTACTAATTTTG-3’; N286Q, 5’-CACCCATCTTAGCTCCATACGGTCCTC-3’. All clones used were derived from a pTracer/hEAG1 construct (3).

Cell Culture and Transfection—CHO-K1 (American Type Culture Collection catalog number CCL-61) and Lecl (American Type Culture Collection catalog number CRL-1735) cell lines were obtained from DSMZ (Braunschweig, Germany) and grown in Ham’s F12 medium or Dulbecco’s modified Eagle’s medium (Invitrogen), respectively, containing 10% fetal calf serum at 37 °C under 5% CO₂. Transfections were carried out using DCC-30 (Eurogentec) or FuGENE (Roche Applied Science) according to the manufacturer’s protocols. Stable transfected cells were selected using 1 μg/ml Zeocin (Cayla). The optimal tunicamycin concentration (2.5 μg/ml; highest concentration allowing survival of ~80% of cells) was determined by the incubation of CHO cells in medium supplemented with decreasing concentrations of tunicamycin (10–0.3 μg/ml). CHO-EAG1 cells were harvested, plated, and allowed to attach for several hours. Culture medium was then replaced by tunicamycin-containing (or Me₂SO-containing) medium without antibiotics. After 24 h of incubation under these conditions the cells were used for further studies. Antisense experiments indicate that the half-life of the protein is 80% of cells) was determined by the incubation of CHO cells in medium supplemented with decreasing concentrations of tunicamycin (10–0.3 μg/ml). CHO-EAG1 cells were harvested, plated, and allowed to attach for several hours. Culture medium was then replaced by tunicamycin-containing (or Me₂SO-containing) medium without antibiotics. After 24 h of incubation under these conditions the cells were used for further studies. Antisense experiments indicate that the half-life of the protein is 24 h in neuroblastoma cells (3).

Immunoprecipitation and Western Blot—5–10 μg of antibody were incubated overnight with 45 μl of protein GA/agarose in PBS with 0.002% bovine serum albumin. Antibody-conjugated beads were washed three times with washing buffer (0.1% Triton X-100, 50 mM Tris-HCL, pH 7.4, 300 mM NaCl, and 5 mM EDTA). To obtain cell lysates, washing buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, and 5 mM EDTA). To obtain cell lysates,
CHO cells were grown to confluency, scraped, washed three times with PBS, and lysed with non-denaturing lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, and 5 mM EDTA). Cell debris was removed by centrifugation, and the supernatants were incubated overnight with antibody-conjugated beads. Protein-antibody-bead complexes were washed three times with washing buffer and used for Western blotting or treated with endoglycosidase. Crude total cell membranes (ER, Golgi, and plasma membranes) were isolated in ice-cold hypotonic solution with protease inhibitor tabs (Roche Applied Science).

Proteins denatured by heat in Laemmli sample buffer were separated by SDS-PAGE (7.5% polyacrylamide gel) and transferred to nitrocellulose filters at pH 10. Nonspecific sites were blocked for at least 30 min with 5% nonfat dry milk in PBS. Membranes were incubated with anti-CHO primary antibody for at least 3 h (room temperature) or overnight (4 °C), washed with PBST (0.1% Tween 20 in PBS), and incubated for 1–4 h with peroxidase-coupled secondary antibody, washed, and developed using enzyme-linked chemoluminescence (ECL kit; PerkinElmer Life Sciences). For differential centrifugation, cells were lysed by osmotic shock in 20 mM Tris-HCl, pH 7.9, 2 mM dithiothreitol, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride supplemented with protease inhibitor tabs (Roche), homogenized 10 times through a 23-gauge needle, and finally sonicated for 5 min on ice. Proteins were stabilized by the addition of 2% sucrose. Intact cells were removed by centrifugation, and the supernatant was collected and further centrifuged at 1,000 (pellet P-1), 10,000 (P-10), and 100,000 × g (P-100). Pellets were resuspended in 40 μl of PBS and tested by immunoblotting.

For surface expression determination, exposed proteins were biotinylated using a cell surface protein biotinylation and purification kit from Pierce. Five 50–60% confluent 16-cm plates of each cell type were incubated for 45 min at 4 °C with 1.8 mg of sulfo-NHS-SS-biotin. Biotinylated proteins were isolated on NeutrAvidin gel columns, eluted by incubation with 200 μl of 50 mM dithiothreitol in SDS-PAGE sample buffer for 10 min at 95 °C, and analyzed by Western blotting.

**Enzymatic Deglycosylation**—Prior to glycosidase digestion, proteins from whole cell lysates were immunoprecipitated with 20 μg of aEag1K62 antibody. Proteins were released from agarose beads by the addition of 20 μl of 0.1 M 2-mercaptoethanol and 0.1% SDS and incubation at 95 °C for 10 min. For the peptide-N-glycosidase F (PNGase F) digestion, 10 μl of immunoprecipitated protein was incubated overnight at 37 °C with 5 units of PNGase F in 25 μl of buffer (60 μM Tris-HCl, pH 8.5, and 0.8% Triton X-100). PNGase F was substituted with 0.5 μg Tris-HCl, pH 8.5, in a control reaction. For endoglycosidase H (EndoH) digestion, 10 μl of immunoprecipitated protein was incubated overnight at 30 °C with 0.5 units of EndoH in 40 μl of buffer containing 75 mM sodium citrate (pH 5.5) and 0.05% phenylmethylsulfonyl fluoride. EndoH was substituted by water in a control reaction. Both reactions were stopped by the addition of Laemmli sample buffer, and products were analyzed by electrophoresis on 7.5% SDS-polyacrylamide gel.

**Immunoprecipitation**—Transiently transfected CHO-K1 or Lec1 cells were grown on coverslips for 2–3 days following transfection. After fixation, permeabilization, and blocking (in 4% paraformaldehyde, 0.1% Triton X-100, and 10% bovine serum albumin, respectively), cells were incubated with the primary antibody and the fluorescently labeled secondary antibody. Nuclei were stained with Hoechst 33342, and coverslips were mounted using the ProLong Gold™ antifade kit and stored at 4 °C. Microscopic analysis was performed using epifluorescence or confocal microscopy. Non-transfected cells did not give any signal with anti-Eag1 antibodies, nor did transfected cells signal when incubated with only the antibody data (not shown).

**cRNA Synthesis and Expression in Oocytes**—cRNA synthesis was performed after Chomczynski and Sacchi (16). When accurate cRNA quantification was required, it was performed using RiboGreen (Molecular Probes) with a ribosomal RNA standard curve.

**Electrophysiology**—Patch clamp (17) measurements were performed at room temperature using an EPC9 amplifier (Heka Electronics). We used pipettes made of aluminum silicate with a resistance of 1–3 MΩ. When filled with the intracellular solution (130 mM KCl, 10 mM Hepes, pH 7.2, and 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(2-aminoethyl)ester). The external solution contained 110 mM NaCl, 30 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose, and 10 mM Hepes. Currents were activated by discrete depolarization to +60 mV from a holding potential of −80 mV, filtered at 1–3 kHz, and sampled at 5–10 kHz. Series resistance was compensated up to 60%. Leak and capacitive currents were subtracted on-line using a Pfn protocol. Current densities were determined for at least five different cells. Cell capacity was determined by the automatic C<sub>∞</sub> function compensation of the amplifier. Oocyte preparation and electrophysiological recordings were performed as described elsewhere (18).

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**RESULTS**

**Eag1 Potassium Channel Undergoes N-Linked Glycosylation**—On immunoblots, a polyclonal antibody directed against the C terminus of rat Eag1 was able to specifically recognize human Eag1 expressed in CHO cells. Two distinct bands, absent in non-transfected cells, were detected in these preparations with electrophoretic mobilities corresponding to ~110 and 130 kDa (henceforth referred to as E110 and E130). Both bands were detected in immunoprecipitates (Fig. 1A) as well as in whole membrane preparations (data not shown). These results are similar to those reported previously for a different antibody (19) and suggest the occurrence of post-translational modifications. We first tested the most common modification, N-linked glycosylation. For this, we studied the effect of removal of either core or complex oligosaccharides using EndoH or PNGase F, respectively, on the electrophoretic mobility of immunoprecipitated Eag1.

After treatment with EndoH, E110 was shifted to ~108 kDa (Fig. 1B), whereas E130 did not show dramatic alterations in its mobility. Because EndoH cleaves only core oligosaccharides, this result indicates that E110 corresponds to a core-glycosylated glycoprotein and not to an alternatively spliced isoform (Eag1b). With this approach we could not determine whether E130 also contains core-glycosylated residues, because a small (2-kDa) change in mobility is beyond the resolution of this method. Nevertheless, we observed a slight shift of E130 compatible with this hypothesis. Removal of all sugar residues by PNGase F altered the mobility of both E130 and E110 and resulted in a single band of ~108 kDa, indicating that both E130 and E110 contain oligosaccharides but that only E130 carries complex forms. The mobility shifts require the presence of the corresponding enzymatic activity (Fig. 1B, lanes marked with a positive sign) and are therefore unlikely because of nonspecific modifications during treatment. In the absence of enzyme (Fig. 1B, lanes marked with a negative sign), the double band pattern of native Eag1 was found unaltered. Proteolytic degradation is also unlikely, because we did not detect lower molecular mass bands. Because no complete shift of the mobility of E130 to that of a non-glycosylated protein was observed after EndoH treatment, we hypothesize that E130 represents a...
complex-glycosylated (and thus EndoH-resistant) and, most probably, post-ER form of Eag1.

Taken together, these data indicate the existence of two distinct glycosylation isomers of the Eag1 protein, E110 and E130. E110 is sensitive to both PNGase F and EndoH and is thus likely to represent a mannose-rich, core-glycosylated Eag1 protein, whereas E130, which is sensitive to PNGase F but insensitive to EndoH, represents a complex-glycosylated form of the Eag1 protein.

In native systems (rat Eag1 isolated from brain tissue), either as total homogenates (data not shown) or as immunoprecipitates (Fig. 1C) the anti-Eag1 antibodies recognized two bands in Western blots with mobilities compatible with ~115 and 130 kDa, similar to those determined for Eag1 expressed in CHO cells. Enzymatic deglycosylation with PNGase F also resulted in a single band of ~108 kDa, compatible with the calculated molecular mass of the unprocessed rat Eag1.

From these results, we concluded that Eag1 can undergo N-glycosylation both in heterologous and native expression systems and that two different oligosaccharides contribute with mobility shifts corresponding to ~2–7 and ~20 kDa to the molecular mass of the mature Eag1.

Subcellular Fractionation of Glycosylated Eag1—Cell extracts were also fractionated by differential centrifugation to enable a crude distinction between large complexes and cytosolic, nuclear, or membrane-bound proteins. CHO cell lysates containing Eag1 were processed by three centrifugation steps as described under “Materials and Methods.” Pellets P-1 (1,000 g), P-10 (10,000 g), and P-100 (100,000 g) were collected and studied by Western blotting (Fig. 2). To monitor the fractionation, GM-130 (a Golgi resident protein) and TRAP (an ER integral membrane protein) were used. Both fractionated mostly within the P-1 and P-10 fractions, and both were only weakly represented in P-100 fraction, which is supposed to contain mainly plasma membrane sheets. The intensity of the band corresponding to TRAP was strong and comparable in both P-1 and P-10 fractions, whereas the signal of GM-130 was weaker and more prominent in the P-10 fraction. As shown in Fig. 2, Eag1 glycosylation isomers were differentially found in the analyzed fractions. Semi-quantitative densitometry analysis showed that the density of the E130 band was nearly the same in all three fractions, with a slight increase in the P-10 fraction. In contrast, the intensity of the E130 was dramatically increased in P-10 and P-100 as compared with P-1. Analysis of the percent contribution of each isoform to the total Eag1 signal in each fraction showed that in the P-1 fraction the E110 form represented a majority (67%) of the Eag1 population in the P-1 fraction, whereas E130 was the prevailing form in P-10 and P-100 (69.7 and 79.1%, respectively; Fig. 2B).

These data suggest that E110 is mainly represented in the P-1 fraction, in which a strong signal of an ER marker and a medium signal of a Golgi marker were detectable. This conclusion agrees with the common observation that core-glycosylated proteins are typical for ER and early Golgi. E130 represented the majority of the Eag1 signal in the P-10 fraction (containing a majority of the Golgi and ER markers), as well as in the P-110 fraction (thought to contain plasma membrane proteins).

Glycosylated Residues of the Eag1 Potassium Channel—Eag1 displays six consensus N-linked glycosylation sites NX(S/T), where X can be any amino acid except proline (20, 21) at asparagines 26, 238, 388, 406, 470, and 752. Following current structural models of voltage-gated channels (22, 23), two of these sites would be located in the intracellular N- and C-terminal domains (Asn-26 and Asn-752) and two in transmembrane regions (Asn-238 and Asn-470). The two remaining potential N-linked glycosylation sites, Asn-388 and Asn-406, are located in the extracellular loop between the fifth transmembrane segment and the pore-lining region H5 and are therefore prime candidates for glycosylation. We substituted each of these asparagine residues with glutamines by site-directed mutagenesis and generated stable cell lines. To minimize the effects of the specific integration site, polyclonal cell lines were used in all further studies.

We determined the electrophoretic migration pattern of each mutant (Eag1-N26Q, -N238Q, -N388Q, -N406Q, -N470Q, and -N752Q) after PNGase F digestion. Mutants on residues not exposed to the extracellular environment (Asn-26, Asn-238, Asn-470, and Asn-752), which are not expected to be glycosylated, showed the typical two-band pattern (110 and 130 kDa) observed for wild type Eag1; glycosidase treatment in these mutants resulted in a single band migrating at ~108 kDa (Fig. 3). This evidence strongly suggests that these asparagine residues are not glycosylated in CHO cells.

In contrast, Eag1-N388Q and -N406Q mutants showed an altered migration pattern. Fig. 3 shows that Eag1-N388Q lacks the E110 band, whereas a single band compatible with E130 is still present. Treatment of Eag1-N388Q with PNGase F resulted in a shift of the E130 band to lower molecular mass (~108 kDa), putatively corresponding to non-glycosylated Eag1. This result suggests that the origin of this band is the same as that of the E130 band of wild type Eag1.

In Eag1-N406Q (Fig. 3), a single 110-kDa band was detectable. This band was also sensitive to PNGase F, because a slight shift in migration (to ~108 kDa) was observed after treatment.

In conclusion, of the six possible glycosylation sites in the Eag1 sequence, only the two predicted sites (Asn-388 and Asn-406) located near the putative pore region are glycosylated. Two different carbohydrate attachments seem to be located on different residues of Eag1, namely EndoH-sensitive core glycosylation at Asn-388 and EndoH-insensitive complex glycosylation at Asn-406.
Double Mutation on Asn-388 and Asn-406 Abolishes Glycosylation—We then generated the double mutant Eag1-N388Q,N406Q, designated NN/QQ, lacking both glycosylation sites. As shown in Fig. 3 (lower right section), the mutant protein migrated at ~108 kDa, corresponding to non-glycosylated Eag1. After overnight incubation with endoglycosidase, we only detected a band migrating at ~75 kDa, suggesting proteolytic degradation of the mutant. It is therefore tempting to speculate that N-linked glycosylation increases the stability and/or protects Eag1 from degradation.

To investigate the nature of the oligosaccharides attached to Eag1 and mutant proteins expressed in CHO cells, we tested the ability of the mutated proteins to bind to concanavalin A (ConA) in pull-down experiments. Both glycosylation isoforms were detectable after the precipitation of wild type Eag1 extracts with ConA (Fig. 4, lane marked wild type). As expected, ConA also pulled down a single 110-kDa band from Eag1-N406Q. However, no band was detected from cells transfected with the N388Q mutant (Fig. 4A), although the presence of Eag1 and mutant proteins in cell lysates was confirmed by Eag1 immunoprecipitation (Fig. 4, section marked Eag1 IP).

These results suggest that the Eag1-N388Q mutant, like other complex-glycosylated proteins, does not contain oligosaccharides that can be recognized by ConA, i.e., α-glucose or α-1,6-mannose. However, Eag1-N406Q, which contains only core-oligosaccharides, is recognized by ConA. ConA did not recognize the complex oligosaccharides attached to Asn-406, but both E110 and E130 are recognized by the lectin in wild type Eag1, indicating that E130 carries oligosaccharides recognizable by ConA. Thus, only E130 is complex-glycosylated at Asn-406, but both E110 and E130 are core glycosylated at Asn-388.

Intracellular Distribution and Surface Expression of Non-glycosylated Eag1—To determine the effects of N-linked glycosylation on the intracellular localization of Eag1, we studied the distribution of the protein in CHO-hEAG1 cells where N-linked glycosylation had been inhibited by tunicamycin treatment. Additionally, an Eag1-expressing cell line (Lec1hEAG1) was generated using Lec1 cells (a CHO-based, glycosylation-deficient cell line). Western blotting revealed in both cases a single band of ~108 kDa, corresponding to the expected size of non-glycosylated Eag1. This finding supports our hypothesis about the identities of E130 and E110 and indicates an effective inhibition of N-linked glycosylation in both systems (Fig. 5, A and B).

Immunofluorescence using monoclonal anti-Eag1 antibodies revealed a spotted, widespread distribution pattern with no remarkable membrane localization in untreated CHO-hEAG1 cells (Fig. 5C). The signal accumulated to perinuclear structures after tunicamycin treatment as well as in Lec1 cells expressing Eag1 (Fig. 5D). We could not find unequivocal colocalization with ER (Trap and p53), Golgi (GM130), endosomes (Rab8 and Rab7), or lysosomal markers (cathepsin D and Limp2) (data not shown). We therefore speculate that those perinuclear structures might correspond to aggresomes (24, 25).

Eag1 wild type and mutant distribution was examined on sucrose gradients using GM-130 as a Golgi marker and calreticulin as an endoplasmic reticulum marker. The non-glycosylated Eag1 NN/QQ localized to the Golgi marker-containing fractions but had almost no colocalization with calreticulin (data not shown).

Intracellular Distribution and Surface Expression of Eag1 Glycosylation Mutants—To determine whether the two distinct N-glycosylation attachments have different effects on Eag1 distribution, we studied the single and double glycosylation mutants in more detail. Indirect immunofluorescence of CHO cells transiently expressing Eag1-N388Q showed a widespread presence of the protein with a patched pattern of the signal distributed throughout the cells and their processes (Fig. 6). This pattern resembles that observed in wild type Eag1. In contrast, cells transfected with Eag1-N406Q and Eag1-NN/QQ displayed an immunofluorescence pattern strongly restricted to compact perinuclear structures, similar to those observed in tunicamycin-treated CHO-hEAG1 or in Lec1-hEAG1 cells (see Fig. 5). These results indicate that N-linked glycosylation at asparagine 406 is important for subcellular distribution of Eag1 and that mutation of this site results in proteins that are intracellularly retained. Mutation of the second glycosylation site (Asn-388) seems to have no dramatic effect on protein distribution.

Surface expression of wild type and mutant channels was further assessed by biotinylation assays on CHO cells. After surface biotinylation on intact cells, labeled proteins were pulled down with streptavidin and immunoblotted. In such experiments (Fig. 6B), Eag1 was biotinylated and could be detected in the blots, whereas Eag1-N406Q was not detectable. These data indicate a dramatic reduction in the amount of protein exposed to the extracellular environment. Similar results were obtained on the double mutant Eag1-NN/QQ, whereas the single Eag1-N388Q mutant showed a similar behavior to wild type, further indicating that the critical glycosylation residue in Eag1 is Asn-406.

Impact of Glycosylation on Functional Properties of Eag1—On the functional level, we analyzed the electrophysiological behavior of Eag1 channels in CHO-hEAG1 and Lec1-hEAG1 cells. Currents measured in the whole cell configuration...
tion of the patch clamp technique can be unequivocally attributed to channels located in the plasma membrane. The average current density (Fig. 7) was dramatically reduced both by tunicamycin treatment and in Lec1hEAG1 cells as compared with untreated CHO-hEAG1 cells. Similarly, cells expressing Eag1-NN/QQ showed a dramatic decrease in current density. This result agrees with immunofluorescent studies indicating that N-linked glycosylation is involved in the mechanism of intracellular Eag1 transport and the arrival of the channels to the cell membrane. However, the reduction in current density was not the only effect, as the remaining current was also kinetically different from that of wild type (see below).

In the Xenopus oocyte expression system, injection of RNA coding for Eag1-N388Q resulted in currents quantitatively very similar to wild type, whereas RNA coding for Eag1-N406Q significantly reduced the current amplitude. This result indicates that complex glycosylation at Asn-406 is required for normal functional expression of the channel, whereas core glycosylation at position 388 has a smaller impact. We expected Eag1-N406Q to have an unaltered ability to form tetramers with wild type channels, because the region determining association would not be affected by this mutation (26). When a 1:1 mixture of Eag1 and Eag1-N406Q RNAs was injected into oocytes, a clear reduction in current amplitude was observed as compared with oocytes injected with wild type channels alone, indicating an association between wild type and mutant channels. The current amplitude in oocytes co-expressing wild type and mutant channels amounted to approximately one-fourth of that elicited by wild type alone (diluted with nuclease-free water). Assuming that the subunit association is random, this means that a single mutant subunit in a tetramer is not sufficient to alter current amplitude (by either altering the trafficking or the functional properties of the channel), because such an effect would render only one-sixteenth of the Eag1 channels remaining active. The observed reduction is compatible with the requirement of at least two mutant subunits in a tetramer to confer the mutant phenotype (in a random combination, 31% of the channel population would be constituted by at least three wild type subunits)

These experiments do not distinguish between a pure effect on protein localization and more direct effects on channel properties. To further address this question, we performed experiments in Xenopus oocytes expressing wild type Eag1 and determined some properties of the current after enzymatic removal of sugar residues with PNGase F. This experimental design allowed us to rule out effects on protein trafficking, because only channels expressed in the plasma membrane are taken into consideration. As seen in Fig. 8, A and B, deglyco-
ylation of the channel protein reduced currents significantly as compared with controls (identical incubation in the presence of heat-inactivated PNGase F), indicating a combination of effects of post-translational modifications both on the channel transport and functional behavior.

As mentioned previously, not only the current amplitude but also the kinetic properties of Eag1 were profoundly affected both by enzymatic deglycosylation and by mutation at position 406. Both conditions cause dramatically slower activation of the channel (Fig. 8, C and D), which is not found by mutation at position 388. In summary, these results indicate that complex glycosylation of Eag1 is not only necessary for normal transport of the protein but also for normal function of the channels already present in the cell membrane.

**DISCUSSION**

Our results show that the ether α-go-go voltage-dependent potassium channel (Eag1, Kv10.1) undergoes heterogeneous N-linked glycosylation, which influences both proper trafficking and functional properties. In both native tissue and transfected cells Eag1 exists in at least two molecular mass forms (∼110 and ∼130 kDa), and each carries N-linked oligosaccharide attachments, as the observed Western blot bands were shifted to lower molecular mass upon enzymatic deglycosylation with PNGase F. Only the 110-kDa band was sensitive to EndoH treatment, supporting the idea that two distinct populations of carbohydrates are attached to Eag1 in CHO cells. Mutation analysis revealed two residues, Asn-388 and Asn-406, as acceptors of N-linked oligosaccharides on Eag1. Both glycosylation sites are located in the predicted extracellular region between the fifth transmembrane segment and the pore domain (22, 23), and each seems to be responsible for one type of oligosaccharide attachment. The core oligosaccharide, sensitive to EndoH and represented by the 110-kDa band, is attached to Asn-388, whereas the complex oligosaccharide is represented by the 130-kDa band and is linked to Asn-406. Both glycosylation sites of Eag1 are modified independently of each other. Thus, mutation of the Asn-388 site does not affect glycosylation of the Asn-406 site and vice versa. Furthermore, lectin-binding assays showed that both glycosylation sites are occupied concomitantly (at least for some population of channels) and that the different forms of glycosylated Eag1 coexist in cells.

Glycosylation of voltage-gated ion channels is frequently reported. In most cases, one or two oligosaccharide attachments are described that vary in size, composition, and location between individual channels and/or different channel families. HERG, the human ether α-go-go gene (Kv11.1), a member of the EAG potassium channel family, shows a similar two-band migration pattern (27, 28) caused by complex and core glycosylation on two active glycosylation sites located in an equivalent region of the protein (29, 30). Glycosylation of the HERG β-subunit (MiRP1) can also determine the affinity of block by pharmacological agents (31). Shaker also shows a banded migration pattern caused by N-oligosaccharide attachments (32). When expressed heterologously in Xenopus oocytes, the Shaker protein is made as an immature, core-glycosylated precursor in the ER, where it...
folds and assembles into tetramers. Upon transfer to the Golgi apparatus and processing of the oligosaccharide residues, the immature protein is converted to the mature structure and delivered to the cell membrane (33–37).

Although N-linked glycosylation is a common modification of plasma membrane proteins, not all ion channels undergo glycosylation. For example, Kv1.6 lacks consensus N-glycosylation sites in the predicted extracellular domains (38). In Kv2.1 the single consensus site is not actively glycosylated, although other members of the same family of proteins (Kv1.1, Kv1.2, and Kv1.4) can undergo glycosylation (38), imparting differential effects depending on the particular channel type (39).

N-Linked glycosylation is important for cell surface expression of many different groups of receptors and ion channels, although the roles of carbohydrate moieties vary between different cases (29, 30, 40–49). N-Linked glycosylation also influences intracellular trafficking of Eag1 potassium channels. Expression of Eag1 in glycosylation-deficient Lec1 cells or tunicamycin-treated CHO cells induces the localization of Eag1 to unidentified perinuclear structures. In contrast, wild type CHO cells transfected with Eag1 showed a dispersed distribution throughout the entire cell, including its processes. Glycosylation at asparagine 406 is apparently necessary for proper intracellular trafficking of Eag1, because both Eag1-N406Q and Eag1-NN/QQ mutants showed peripheral accumulation similar to the pattern observed in glycosylation-deficient cells. Conversely, the distribution pattern of the Eag1-N388Q mutant was similar the one observed for wild type Eag1.

In Xenopus oocytes, disturbance of the complex glycosylation at Asn-406 by either mutation or enzymatic treatment resulted in a dramatic reduction (~6.5-fold) of the average peak current amplitude in comparison with wild type channels. The reduction in current amplitude induced by lack of glycosylation at position 406 is, however, not complete. This could be due to some channel molecules escaping the sorting controls but also to an association to endogenous proteins that makes it possible for the channels to reach the cell surface. Immunocytochemical observations indicate that complex glycosylation at Asn-406 is necessary for normal distribution of Eag1 in CHO cells. However, we have also observed that enzymatic removal of oligosaccharides from channels already expressed in the membrane induces the same functional effects as the mutation at Asn-406, indicating that aside from altered channel distribution, even channels that reach the plasma membrane in the mutant undergo a profound alteration in their functional properties.

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