Mapping the Kidney Potassium Channel ROMK1
GLYCOSYLATION OF THE PORE SIGNATURE SEQUENCE AND THE COOH TERMINUS*

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ROMK1, also known as Kir 1.1, is an inwardly rectifying K⁺ channel and is the prototypical member of the large Kir gene family. The accepted model of Kir topology predicts intracellular NH₂ and COOH termini, and two membrane-spanning segments, M1 and M2, connected by an intramembranous pore-forming segment, H5. The sequence of H5 is similar in voltage-dependent K⁺ channels and features a strictly conserved GY/FG in its mid-region, which has been proposed as the selectivity filter of the pore. We have been using N-glycosylation substitution mutants to map the extracellular topology of ROMK1 biochemically and have described several loci in H5 that were glycosylated. We now report glycosylation at loci Tyr¹⁴⁴ and Phe¹⁴⁶, which indicates that the signature GYG sequence (143–145) rather than being intramembranous is extracellular. The COOH terminus was predicted to begin at position 178, but contrary to the model, we observed that position 257 was glycosylated and surrounding positions at 199, 222, and 298 were unglycosylated. N-Glycosylation sequence substitution at the latter three positions abolished K⁺/Na⁺ selectivity. Our results suggest a major revision of the topology of ROMK1 with H5 and the pore signature sequence now completely extracellular. The COOH terminus appears to form two additional membrane-spanning segments and to contribute to the ion conduction pathway.

The topological model of ROMK1 and related members of the Kir gene family was predicted from hydropathy plots and, in the case of H5, from sequence similarity to Kv channels (Fig. 1A). The NH₂ and COOH termini were cytoplasmic, based on the absence of a signal sequence in the open reading frame, and the α-helical transmembrane segments M1 and M2 were separated by a linker containing a 17-residue stretch called H5 (9–13). In Kv channels, H5 is thought to form an intramembranous hairpin structure with the selectivity filter at the highly conserved GYG, which is at loci 143–145 of ROMK1. Recently, we showed that several H5 loci of ROMK1 could be glycosylated and that one of them, Q139N, in both its glycosylated and unglycosylated forms was nonselective between K⁺ and Na⁺ (14). That H5 is a hotspot for residues that affect the pore is supported by the report that Leu¹¹⁷ and Val¹²¹ in ROMK2, corresponding to 136 and 140 in ROMK1, retained K⁺ selectivity but increased single channel conductance and Ba²⁺ sensitivity (15). However H5 is not the sole pore-determining segment, since most of the important determinants of pore blockade by Mg²⁺ and polyamines are negatively charged residues located in the carboxyl terminus (16–19) and M2 (20–24).

Our previous results (14) suggested that H5 and its flanking regions E1 and E2 formed the first extracellular loop (EL1) of Kir’s and that the second transmembrane segment (M2) was shifted toward the COOH terminus (Fig. 1B). The possibility remained that the GYG signature pore sequence was intramembranous, and to test this, we engineered N-glycosylation substitution mutants (GSMs), which are markers of extracellularity in and around this region. We also targeted the COOH terminus thought to become cytoplasmic at position 178 for this type of evaluation. Glycosylation was demonstrated by gel shift assays and reductions in ROMK1 currents before and after tunicamycin (TM) treatment. Our results show that the present topological model requires major revision. We found that position 144, supposedly forming the intramembranous selectivity filter of the pore, was glycosylated as was its immediate neighbor at locus 146. Thus the signature sequence rather than being intramembranous is extracellular. We found that position 257 was glycosylated, whereas surrounding loci at 199, 222, and 298 were not. However, N-glycosylation site substitution at 199, 222, and 298 rendered ROMK1 nonselective for K⁺ over Na⁺. It appears that the COOH terminus contributes at least two additional transmembrane segments to Kir topology and may form part of the ion conduction pathway.

EXPERIMENTAL PROCEDURES

Materials—TNM-FH insect medium was purchased from JRH Biosciences; Sf9 cells were obtained from American Type Culture Collection for publication.

The abbreviations used are: Kv channel(s), voltage-gated K⁺ channels; GSM(s), N-glycosylation substitution mutant(s); Sf9, Spodoptera frugiperda; TM, tunicamycin; PBS, phosphate-buffered saline; WT, wild type; MES, 4-morpholineethanesulfonic acid.

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tion; the TA cloning kit was from Invitrogen; the BaculoGold™ transfection kit was from PharMingen; fetal bovine serum and penicillin-streptomycin and pluronic F-68 10% solution were from Life Technologies, Inc.; Zwittergent 3–10 was from Calbiochem; Tween 20, tunicamycin, phenylmethylsulfonyl fluoride, aminobenzamidine, bovine serum albumin, and trypsin blue solution were from Sigma; and Qiagen columns were from Qiagen.

**Cell Culture and Recombinant Baculoviruses—**Sf9 cells were grown in Hink's TNM-FH insect medium containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1% pluronic F-68 under natural atmosphere at 27 °C. Cells were maintained in monolayer cultures and passed every 4–10 days. Production of recombinant protein was conducted in suspension cultures at a cell density of 1–1.2 × 10⁶ cells/ml using viral supernatant from suspension culture amplifications. The recombinant baculoviruses were generated by cotransfection of Sf9 cells with baculovirus vectors containing the ROMK1 for immunooaffinity purification, Western blots, and immunocytchemistry.

**Glycosylation of the Selectivity Filter and COOH Terminus of ROMK1**

**Experimental Approach**

In our topological model of ROMK1, the H5 segment thought to be intramembranous was proposed as extracellular (Fig. 1B). To address experimentally the extracellularity of both the GYG branched structure within H5 and the putative intracellular COOH terminus, we used N-glycosylation tagging. All of the GSMs have the native N-glycosylation site removed (NRT, N-glycosylation sequon (26). Sf9 cells were infected with Anaxclump IB amplifier (Axon Instruments, Foster City, CA) with membrane capacitance and resistance compensations. Fire-polished pipette electrodes had tip resistances of 2–5 and 5–10 MΩ for whole-cell and single channel measurements, respectively. Voltage pulse protocols and data acquisition and analysis were carried out using the pClamp suite of profrags (Axon Instruments). Data were filtered at 1 kHz and subsequently digitized at 5 kHz.

**RESULTS**

**Detection of Glycosylation by Structural and Functional Assays**

**Gel Shift Assays—**Proteins were immunooaffinity-purified and detected by Western blotting using the M2 FLAG epitope fused to the NH₂ terminus (WT ROMK1, Y144N/F146S, V199N, I222N, N259T, and D298N) or both termini (F146N/F148S). The octapeptide attached to the NH₂ terminus (26) or both termini did not interfere with WT ROMK1 function. Pre-
glycosylation sequons were similar, indicating that the Ba$^{2+}$ block was no longer time-dependent (Table I). In addition, the outward currents for V199N, I222N, and D298N were blocked (Fig. 5B).

**Cellular Location of ROMK1 H5 Substitution N-Glycosylation Mutants**

The small currents for Y144N/F146S suggested that either lower amounts were expressed at the cell surface or fewer channels were in the open state at the cell surface. To distinguish between these possibilities, ROMK1 mutant proteins were localized in Sf9 cells using the M2 monoclonal antibody and fluorescence immunocytochemistry in combination with laser confocal microscopy. The immunofluorescence staining patterns for the mutants were similar, and the protein appeared to be at the cell surface in the majority of the cells. The thin outer immunofluorescence ring for both Y144N/F146S and F146N/F148S indicated that the channel proteins were at the cell surface as we described for WT ROMK1 (Fig. 6). Immunofluorescence was also observed as a thicker inner ring around the nucleus and patches in between the rings. The immunofluorescence around the nucleus was attributed to pro-
FIG. 3. Reduction in whole-cell ROMK1 currents produced by tunicamycin. Whole-cell currents of ROMK1 GSMS were reduced (A) or unaffected (B) by TM. Only glycosylated GSMS were affected by TM. Mean current-voltage relationships, plus (filled squares) and minus (open squares) TM, are on the far right. Currents were elicited by voltage steps from −120 to +90 mV in 20-mV increments from a holding potential of 0 mV in a solution containing (in mM): potassium aspartate, 140; MgCl₂, 1; MES, 10; mannitol, 60 (pH 6.3, adjusted with Tris-OH). The pipette solution contained (in mM): potassium aspartate, 140; MgCl₂, 5; HEPES, 10; EGTA, 10; mannitol, 40 (pH adjusted to 7.2 with Tris-OH). The number of cells expressing the SMs, in the absence and presence, respectively, of TM are: F146N/F148S, 7 and 6; N259T, 10 and 10; V199N, 4 and 2; I222N, 2 and 2; D298N, 7 and 4.

FIG. 4. Single channel recordings of glycosylated ROMK1 N-glycosylation substitution mutants. Representative single channel recordings of cells expressing F146N/F148S and N259T, plus and minus TM. On the right, the opening probability (Pₒ) was plotted for cells with and without TM. Single channel currents in the cell-attached mode were elicited by 350-ms steps at −100 mV from a holding potential of 0 mV, at 1 Hz. Pipette and perfusion solutions were similar to the extracellular solution as indicated in the legend to Fig. 3. For F146N/F148S, the traces were averaged from 283 and 229 sweeps and for N259T traces were averaged from 198 and 398 sweeps, in the absence and presence of TM, respectively.
TABLE I
ROMK1 N-glycosylation mutants and their function

| Glycosylation | Surface expression | Function | Whole-cell reduction \( \pm \) TM (%) | Single channel \( P_e \) \( \pm \) TM | \( \Delta V \) | \( I_{peak}/I_{steady} \) (BaCl\(_2\)) |
|---------------|--------------------|----------|----------------------------------------|---------------------------------|---------|-----------------|
| WT           | +\(^a\)            | +\(^a\)  | -75\(^a\)                              | 0.8\(^a\)                       | 0.25\(^a\) | 61.6 \(\pm\) 2.3 | 3.1 \(\pm\) 0.7 |
| N117Q        | -\(^a\)            | +\(^a\)  | 6.5 \(\pm\) 43                         | 0.22 \(\pm\) 0.03               | 0.22 \(\pm\) 0.03 | 38.9 \(\pm\) 2.9 | 2.2 \(\pm\) 0.23 |
| Y144N/F146S  | +                  | +        | -89 \(\pm\) 6.4                        | 0.93 \(\pm\) 0.02               | 0.09 \(\pm\) 0.03 | 51 \(\pm\) 5.5 | 2.2 \(\pm\) 0.3 |
| F146N/F148S  | +                  | +        | 9.1 \(\pm\) 30                         | 0.04 \(\pm\) 0.01               | ND\(^b\) | 0.51 \(\pm\) 1.1 | 0.96 \(\pm\) 0.04 |
| V199N        | -                  | +        | 6.6 \(\pm\) 24                         | 0.03 \(\pm\) 0.02               | ND\(^b\) | -0.5 \(\pm\) 0.5 | 0.93 \(\pm\) 0.04 |
| I222N        | -                  | +        | -72.8 \(\pm\) 7.4                     | 0.84 \(\pm\) 0.03               | 0.05 \(\pm\) 0.02 | 48.3 \(\pm\) 1.7 | 2.2 \(\pm\) 0.52 |
| N259T        | +                  | +        | 2 \(\pm\) 28                           | 0.05 \(\pm\) 0.02               | ND\(^b\) | 1.5 \(\pm\) 0.81 | 1 \(\pm\) 0.03 |
| D298N        | -                  | +        |                                       |                                 |         |                 |                 |

\(^a\) Ref. 26.
\(^b\) Ref. 14.
\(^c\) ND, not determined.

The percent reduction in the whole cell currents was the difference in the currents of GSMs with and without TM, divided by currents of GSMs without TM treatment, at \(-120\) mV. The single channel opening probability \(P_e\) at \(-100\) mV in cells with and without TM. The single channel conductance was obtained at voltage steps from \(-180\) to \(-40\) mV at 20-mV increments, from a holding potential of 0 mV. The \(\Delta V\) represents the change in the potential of the zero membrane current when the solution perfusing the cell was changed from high to low K\(^+\). The time dependency of the barium block was expressed as the ratio between the level of the current at the beginning (peak) and end (steady-state) of a 200-ms pulse at \(-120\) V.

**FIG. 5.** K\(^+\)/Na\(^+\) selectivity and Ba\(^2+\) block of ROMK1 N-glycosylation substitution mutants. A, whole-cell currents recorded in a high K\(^+\) solution (left side); in mM: potassium aspartate, 140; MgCl\(_2\), 1; MES, 10; mannitol, 60 (pH 6.3, adjusted with Tris-OH) and low K\(^+\) solution (center); in mM: sodium aspartate, 135; potassium aspartate, 5; MgCl\(_2\), 1; MES, 10, mannitol, 60 (pH 6.3, adjusted with Tris-OH). Current-voltage relationships of the mutants are shown on the right side. Open and filled circles are high and low K\(^+\) solutions, respectively. B, whole-cell currents in the high K\(^+\) solution before and after addition of 7 mM BaCl\(_2\). The first 50 ms of the recordings are shown. Currents were evoked by voltage steps from +80 to \(-100\) mV, from a holding potential of 0 mV.

**DISCUSSION**

N-Glycosylation Substitution Sequons in ROMK1—N-Linked oligosaccharides were detected for GSMs at positions 144, 146, and 257 (Fig. 7). Further evidence of glycosylation was provided by the gel shifts produced by TM. The different amounts of glycosylated monomer for WT ROMK1, Y144N/F146S, F146N/F148S, and N259T, and also GSMs from our previous study (14), reveal differences in the accessibility of the native and novel sites to the oligosaccharyltransferase. Since utilization of an N-glycosylation site depends on the placement of a site in the exoplasmic loop, the distance from the NH\(_2\) terminus, and the conformation of a tripeptide sequon (28–30), the degree of glycosylation may differ. Glycosylation was also consistent with the large reductions in whole-cell currents caused by TM, which correlated with large reductions in \(P_e\) rather than decreases in channel number as noted previously (14, 26). In addition, no apparent difference in channel density at the cell surface of F146N/F148S in the presence and absence of TM was observed by immunocytochemistry measurements.

The present results with TM along with our previous studies (14, 26) show that at least one glycosylated subunit was incorporated into the functional channels at the cell surface. In addition, immunocytochemistry of Y144N/F146S and F146N/F148S showed that channels were transported to the plasma membrane. Taken together, we conclude that positions 144, 146, and 257 were glycosylated and assembled as functional channels. We also note that N-linked oligosaccharides positioned anywhere along the ROMK1 sequence stabilized the open state of the channel, consistent with our previous studies.
The significance of frequently stabilize the open state of the glycosylated channels. A possible mechanism of the unglycosylated channels was distinct any case, our results do indicate that neither the side chains at positions 144, 146, and 259 nor the bulk of the carbohydrate at positions 199, 222, and 298 were mutated. However, three mutations in the intracellular COOH terminus at positions 199, 222, and 298 produced a time-independent block. This result is consistent with a more accessible binding site or a higher affinity of Ba\(^{2+}\) for the V199N, I222N, and D298N channels. Thus, mutations in the COOH terminus segment of ROMK1 appeared to alter the structure or entrance of the pore.

**Significance for Topology**—Our results show that the GYG signature pore sequence and part of the putative intracellular COOH terminus are extracellular, suggesting two additional transmembrane segments (Fig. 7). The recent results, together with our previous demonstration that positions 117, 128, 133, 139, and 153 could be glycosylated (14), places the signature GYG sequence and the linker between M1 and M2 (EL1) as extracellular (Fig. 7). In contrast to earlier models (Fig. 1), we also provide evidence of glycosylation at position 257, indicating that part of the putative cytoplasmic COOH terminus is extracellular. Furthermore, the lack of utilized sites at positions 199, 222, 274, 298, 377, and 384 (14) and the presence of protein kinase A phosphorylation sites at positions 219 and 313 (34) would indicate that there are at least two transmembrane segments in the putative cytoplasmic COOH terminus. Based on these findings and glycosylation studies, which have indicated that a utilized N-glycosylation sequon is a minimum of 11 residues from the nearest transmembrane segment and in an exofacial loop of at least 30 residues (29, 30), we predict that the 10–20 residues, depending on whether the transmembrane segments are \(\alpha\)-helices or \(\beta\)-strands, that make up M3 would range from 222 to 246 and those for M4 from 270 to 308 (Fig. 7). Other experimental results that would be consistent with our proposed model are the following: NH\(_2\) terminus involved in regulating intracellular pH sensitivity (35, 36); M1-H5 region implicated in external pH regulation (37), Ser\(^{44}\) site for cAMP-dependent kinase phosphorylation (34); the Walker A-type motif, running from Gly\(^{23}\) to Lys\(^{229}\) (38); and position 171 involved in Mg\(^{2+}\) and polyamine binding (20, 21).

Our study has also shown that mutating residues Val\(^{199}\), Ile\(^{222}\), and Asp\(^{298}\) with Asn produced nonselective channels and a time-independent barium block. In our newly revised model these residues are localized in or near the membrane where they may contribute to the selectivity filter. Thus, residue Asp\(^{298}\) may be located near M4. Ile\(^{222}\) may be near the membrane or may be part of M3. In our model Val\(^{199}\) is part of IL1, because Ser\(^{210}\) was shown to be phosphorylated. However, this segment may either interact with the membrane or may loop into the cytoplasmic entrance of the pore. Clearly, future studies are needed to determine the exact placement of these residues with respect to the plasma membrane and to determine whether these residues are directly involved in forming the selectivity filter and barium binding sites or whether they are indirectly involved in stabilizing the pore structure.

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**FIG. 6. Cellular localization of H5 Mutants.** Confocal images of permeabilized infected S9 cells expressing Y144N/F148S (A) and F146N/F148S (B). Cells were fixed with paraformaldehyde, incubated with the M2 antibody (1:100), directed against the M2 epitope fused to the NH\(_2\) terminus of the protein, and subsequently incubated with the secondary rhodamine-conjugated sheep anti-mouse antibody (1:50). The white arrows indicate protein in the plasma membrane.

**FIG. 7. Revised topological model of ROMK1 as predicted from N-glycosylation tagging.** The NH\(_2\) terminus, M1, EL1, and M2 are similar to our model shown in Fig. 1B. The recent revision shows that the putative intracellular COOH terminus contributes two additional transmembrane segments, M3 and M4, a second extracellular loop, EL2, and an intracellular loop, EL1. M3 ranges from residue 222 to residue 246 and M4 from 270 to 308, based on glycosylation at position 257 and phosphorylation at Ser residues 219 and 313. The underlined numbers represent the first residues of the introduced N-glycosylation sequons from this study and other numbers are from our previous reports. The branched structure denotes glycosylation. The Ser residues are cAMP-dependent kinase sites (34).
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