Site-directed Glycosylation Tagging of Functional Kir2.1 Reveals that the Putative Pore-Forming Segment is Extracellular

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Running Title: Glycosylation of the Putative H5 and Pore Signature Sequence of Kir2.1
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Summary

Inwardly rectifying K⁺ channels or KirS are a large gene family and have been predicted to have two transmembrane segments, M1 and M2, intracellular N- and C-termini, and two extracellular loops, E1 and E2, separated by an intramembranous pore-forming segment, H5. H5 contains a stretch of eight residues that are similar in voltage-dependent K⁺ channels, Kvs, and this stretch is called the signature sequence of K⁺ channels. Because mutations in this sequence altered selectivity in Kvs, it has been designated as the selectivity filter. Previously, we used N-glycosylation substitution mutants to map the extracellular topology of a weak inwardly rectifying K⁺ channel, Kir 1.1 or ROMK1, and found that the entire H5 segment was extracellular. We now report utilization of introduced N-glycosylation sequons (NXS/T) at positions Ser¹²⁸ in E1, and Gln¹⁴⁰, Ileu¹⁴³ and Phe¹⁴⁷ in the H5 sequence of a strong inwardly rectifying K⁺ channel, Kir 2.1. Furthermore, we show that biotinylated channel proteins with N-linked oligosaccharides attached at positions 140 and 143 in the signature sequence are at the cell surface. Mutant channels were functional as detected by whole-cell and single channel recordings. Unlike Kir1.1, position Lys¹¹⁷ was not occupied. We conclude that for yet another K⁺ channel, the invariant GY/FG sequence is extracellular rather than intramembranous.
Introduction

Kir2.1, also known as IRK1, is a member of a large gene family of inwardly rectifying K⁺ channels, Kirs (1-3). These channels conduct current more efficiently in the inward than the outward direction due to a voltage-dependent block by intracellular Mg²⁺ and polyamines, and have high selectivity for K⁺ over Na⁺ (1, 2, 4-6). Kirs differ in the degree of rectification and in single channel conductance (3). Physiologically, Kirs maintain the resting membrane potential, regulate cellular excitability, and secrete K⁺ necessary for either K recycling or K excretion.

Based on hydropathy plots, Kirs are predicted to have two α-helical transmembrane segments M1 and M2, and cytoplasmic N- and C- termini (Figure 1A, Left Panel). The putative pore-forming segment H5 is thought to be intramembranous and links the two extracellular loops, E1 and E2. Voltage-dependent K⁺ channels, Kvs, have a highly homologous H5 segment that appears to be the major pore determinant (Figure 1B, 7-10). H5 contains eight highly conserved residues and has been proposed as the selectivity filter of K⁺ channels (Figure 1B: 9). Three residues in the signature sequence GY/FG are absolutely conserved among all K⁺ channels. The prediction of K selectivity by H5 is in agreement with the crystal structure of a K⁺ channel, KcsA from Streptomyces lividans (11) in which five residues, TVGYG, of the signature sequence are critical for selective permeation. In this structural model, residues TVGYG of KcsA, as well as the corresponding residues of other K⁺ selective channels, are proposed to orient their side chains away from the pore so backbone carbonyl oxygen atoms form the selectivity filter, and thus GYG are absolutely required for K⁺ selectivity.

However, for Kir6 and eag K⁺ channel families, the accuracy of this general K-selective pore design was challenged because a Phe is substituted at the Tyr position of the
GYG sequence (Figure 1B). In addition, a recent study of KcsA has shown that this same nonconserved substitution does not alter electrophysiological properties (12). Two recent studies of Kir2.1 also demonstrate that K⁺ selectivity was retained when Tyr was mutated to Phe (13), and the amide carbonyls of the invariant Gly residues were changed to ester carbonyls (14).

We have mapped the topological structure of Kir1.1, a weak inwardly rectifying K⁺ channel, using biochemical methods to identify which novel N-glycosylation sequons of Kirs are utilized (15-17). The initial steps of N-glycosylation occur on the luminal side of the endoplasmic reticulum, and therefore only extracytoplasmic segments of membrane proteins can be glycosylated (18). Our findings for Kir1.1 were inconsistent with the accepted Kir topological model because positions 117, 128, 133, 139, 144, 146 and 153, between M1 and M2, including the signature sequence could be glycosylated. The results indicate that H5 is part of the extracellular loop between M1 and M2, and does not penetrate the membrane (15-17). To assess whether this biochemically-derived topological structure is an accurate description of other Kirs, we examined a different member of the Kir class, Kir2.1 (Figure 1B). These two distinct inward rectifiers were chosen based on their different biophysical properties; Kir2.1 is a strong inwardly rectifying channel and exhibits intrinsic gating, whereas Kir1.1 is a weak inwardly rectifying channel and lacks time-dependent gating (3). Kir2.1 N-glycosylation substitution mutants were engineered to contain one novel site. Glycosylation was demonstrated by immunoband shift assays before and after, either tunicamycin (an inhibitor of N-glycosylation), or endoglycosidase H (an enzyme which cleaves high-mannose oligosaccharides from glycoproteins) treatment. Cell surface expression was detected by biotinylation of surface proteins and whole cell and single channel currents. We found that introduced N-glycosylation sites at positions 140 and 143, in
the middle of the signature sequence, were utilized and both glycoforms were present in the plasma membrane. Positions 128, E1, and 147, H5, were also glycosylated (Figure 1A, right panel). All mutant proteins produced functional channels at the cell surface. Taken together our findings indicate that H5, as well as the signature sequence, of the Kir class of K+ channels is topologically extracellular.
Materials and Methods

**Materials**- Sf9 (*Spodoptera frugiperda*) cells were obtained from American Type Culture Collection. TA cloning kit was purchased from Invitrogen, TNM-FH insect medium from JRH Biosciences, BaculoGold™ transfection kit from Pharmingen, fetal bovine serum, and pluronic F-68 10% solution from Gibco Life Technologies, Zwittergent 3-10 from Calbiochem, Tween 20, tunicamycin, phenylmethylsulfonylfluoride, aminobenzamidine, bovine serum albumin, gentamicin, M2 resin, M2 antibodies and trypan blue solution from Sigma, endoglycosidase H from New England Biolabs, and QIAGEN columns from QIAGEN.

**Mutagenesis**- Polymerase Chain Reaction (PCR) was used to fuse the oligonucleotide encoding the M2 FLAG epitope (DYKDDDDK) to the N-terminus of Kir2.1 cDNA. All Kir2.1 N-glycosylation substitution mutants were engineered by PCR overlap extension using FLAG/Kir2.1 cDNA as template (15). PCR products were subcloned into pCRII and pCR2.1 for amplification and sequencing, and subsequently subcloned into either NotI/BamHI double-digested baculovirus transfer vector (pVL1392) or EcoRI digested baculovirus transfer vector (pACSG2). Standard procedures were followed for DNA amplification, isolation and sequencing (19).

**Cell Culture and Recombinant Baculoviruses**- Sf9 cells were grown under natural atmosphere at 27°C in Hink’s TNM-FH insect medium containing 10% (v/v) fetal bovine serum, 10 µg/ml gentamycin and 0.1 % pluronic F-68. Cells were maintained in monolayer cultures and passaged every 3-7 days. Recombinant proteins were produced in suspension cultures at a cell density of 1-1.2 x 10^6 cells/ml using viral supernatant from suspension culture amplifications. The recombinant baculoviruses were generated by co-transfection of
Sf9 cells with baculovirus vectors encoding WT Kir2.1 and Kir2.1 N-glycosylation substitution mutants cDNAs, and BaculoGold viral DNA (modified AcNPV) according to the manufacture’s instructions (Pharmingen). Monolayer cultures were infected with the co-transfected viral supernatant to prepare viral seed stocks. High viral titers were produced by infecting suspension cultures (0.5-0.9 x 10^6 cells/ml) with viral seed stocks. The viral supernatant and the infected cells were collected 3 to 5 days and 1-3 days after infection, respectively. Occupancy of introduced N-glycosylation sites was verified by treating cells with tunicamycin. Tunicamycin (25 µg/ml) was added to infected cells in suspension cultures about 15 minutes after inoculation with viral supernatant.

*Western Blot Analysis*- Infected Sf9 cells (4 x 10^7 to 5 x 10^7) were harvested approximately 24-48 hours post-infection (p.i.) by centrifugation at 2000 x g for 10 minutes. Cell pellets were washed with PBS three times and then resuspended in solubilization buffer (50 mM sodium phosphate monobasic, pH 7.4, 0.3 M KCl, 2% Zwittergent 3-10 or triton X-100; 1.5ml), plus 80 µl of the M2 antibody affinity gel (Eastman Kodak) resuspended in PBS. M2 is an anti-mouse monoclonal IgG that recognizes the FLAG epitope attached to Kir2.1 proteins. The sample was gently rocked for 1 hour at room temperature. Resin was washed thrice with the solubilization buffer without the detergent (1ml) and then washed three times with PBS. Beads were resuspended in 125-150 µl reducing SDS-PAGE sample buffer (2x) and either heated at 90°C for 4 minutes or incubated overnight at room temperature, and then diluted appropriately with SDS-PAGE sample buffer (1x) prior to separation on 12% SDS-polyacrylamide gels. The electrophoresed proteins were transferred to Immobilon P membranes and probed with the M2 antibody (16). The gels were run until the 46 kDa
rainbow marker was 0.25-2.5 cm from the bottom of the gel. Only the sections of the gels that contain Kir2.1 proteins are shown.

Biotinylation of Surface Proteins- Infected cells were harvested (2.2 x 10⁷ or 4.4 x 10⁷) after 24 hours post-infection by spinning cells at 2000 x g for 10 minutes at 4°C. Cells were washed three times with ice-cold PBS (pH 8.1, 100 sodium phosphate monobasic, 0.15 M NaCl). Cell pellet was resuspended in 1 mL ice-cold PBS (pH 8.1). Added 0.7 mg sulfo-NHS-biotin (Pierce) per ml of reaction volume and gently rocked reaction at room temperature for 30 minutes. Washed biotinylated cells three times with ice-cold PBS (pH 8.1) to remove biotinylation reagent. Biotinylated cells were resuspended in 1.5 mL ice-cold PBS (pH 7.5, 20 mM sodium phosphate monobasic, 0.5 M NaCl, 2% triton x-100/zwittergent 3-10). Sonicate sample for 30-60 seconds to break up large cell debris and shear genomic DNA, and subsequently incubated sample on ice. Add 80 µl strepavidin-agarose to sample. Incubate sample at room temperature for 1 hour on a rotator at room temperature and then wash resin 3 times with PBS (pH 7.5, 20 mM sodium phosphate monobasic, 0.5 M NaCl). For endoglycosidase H treatment, resin was washed three times with endoglycosidase H buffer. Reaction volume of endoglycosidase H reaction was 100 µL, plus or minus 2 µl endoglycosidase H. Wash sample two times with PBS (pH 7.5). Resuspend resin in 100 µl 2x SDS-PAGE sample buffer. Heat samples to 95°C for 3 minutes.

Patch-Clamp Recordings- Infected cells from suspension cultures were added 20-24 h p.i. to Petri dishes, containing glass coverslips, and subsequently analyzed at room temperature for up to 72 h p.i. Cells (about 1.25 x 10⁶) for patch clamp analysis were also directly infected on Petri dishes containing glass coverslips with similar incubations times and temperatures. Currents were measured at room temperature using an Axopatch 200A
amplifier (Axon Instruments) with membrane capacitance and resistance compensations in cells. Fire-polished pipette electrodes had tip resistances of 3-10 MΩ for whole-cell recordings and 5-11 MΩ for single channel recordings. The pClamp6 suite of programs (Axon Instruments) was used for voltage pulse protocols, data acquisition and analysis. Data were filtered at 1 kHz, and subsequently sampled at 2 kHz for whole cell recording and 10 kHz for single channel recording. Group data are presented as mean ± SD (number of observations). Origin 4.0 (Microcal Software) was used for statistical analysis and graphics.
Results

Experimental Design

In our topological model of Kir1.1, the putative H5 segment is extracellular (15, 16). To assess whether the extracellularity of this segment is novel to Kir1.1 or is a general structural feature of Kirs, we have studied a strong inwardly rectifying K⁺ channel, Kir2.1, using N-glycosylation tagging. N-glycosylation sites were introduced one at a time in and near the pore-forming segment, H5, see Figure 1A, left panel. Each Kir2.1 N-glycosylation substitution mutant was designed to contain one unique site to minimize disruptions in the protein. The native consensus N-glycosylation site (NET, amino acid residues 190-192) was not removed because it is not utilized (see below, Figure 2). Recombinant proteins were expressed in Sf9 cells infected with recombinant baculovirus encoding the Kir2.1 N-glycosylation substitution mutants. Glycosylation was demonstrated by immunoband shift assays before and after tunicamycin treatment. Whole-cell and single channel recordings evaluated channel structure and surface expression. Cell surface expression of glycosylated channel protein was detected by biotinylation of surface proteins, and subsequently by immunoband shift assays, in the presence and absence of endoglycosidase H digestion.

Detection of Glycosylated Protein from Total Membranes

Proteins were immunoaffinity purified and detected by Western blotting using the M2 FLAG epitope fused to the N-terminus (WT Kir2.1, K117N, S128N, Q140N, I143N/Y145T and F147N/C149T). One band was observed for WT Kir2.1 at approximately 48 kDa, as well as for K117N (Figure 2). The observed molecular weight was similar to the calculated molecular weight of 48,497 Da. Two bands were detected for S128N, Q140N, I143N/Y145T
and F147N/C149T. The lower band was similar to that of WT Kir2.1 while the upper band migrated to about 51 kDa.

When cells were treated with tunicamycin, an inhibitor of N-glycosylation (20), only the lower band at 48 kDa was present for WT Kir2.1 and Kir2.1 N-glycosylation substitution mutants (Figure 2). The absence of a band shift for WT Kir2.1 confirms that the native N-glycosylation sequon is not occupied, and that the introduced site at position 117 was not utilized. Removal of the upper band for S128N, Q140N, I143N/Y145T and F147N/C149T indicate that these sites are utilized. The ratio of glyco- to aglyco-forms differed with placement of the introduced N-glycosylation site. Novel sequons at positions 140, in the middle of H5, and 147, tail of H5, were more accessible to the oligosaccharyltransferase than position 128, site in E1, and much more than position 143, the central residue in the signature sequence.

Functional Channels at the Cell Surface

Whole cell recordings of infected Sf9 cells expressing WT Kir2.1 show inward noninactivating currents with hyperpolarizing pulses (Figure 3A, upper-left panel). The outward currents at pulses more positive to the zero-current potential (0 mV) were much smaller than the inward currents for the opposite driving force which is a feature of Kir2.1, a strong inward rectifier. Similar current patterns were observed for K117N, S128N, Q140N, I143N/Y145T and F147N/C149T (Figure 3A, left panel). However, the currents for Q140N and I143N/Y145T were much smaller. In control experiments, currents recorded from Sf9 cells infected with recombinant baculovirus, encoding the N-terminus fragment of H, K-ATPase α2A (amino acid residues 1-110), were smaller than typical whole-cell currents of Q140N and I143N/Y145T (Figure 3B). When expression levels were low for F147N/C149T
(Figure 3B), as well as WT Kir2.1 and other Kir2.1 mutants, the whole-cell currents were small and the patterns were similar to those of Q140N and I143N/Y145T. However, small currents were atypical for WT, K117N, S128N and F147N/C149T, and due to a combination of low viral titers and short post-infection times (less than 22 h).

Functional single channel currents were recorded, prior to the whole cell currents in some of the infected cells expressing WT Kir2.1 and Kir2.1 N-glycosylation substitution mutants, as shown in Figure 3A, right panel. Currents of WT Kir2.1, as well as K117N, S128N and F147N/C149T, had long-lived openings and closures. Current amplitudes at –100 mV for K117N and S128N were similar to WT Kir2.1, while that of F147N/C149T was about 45% of the main conductance. Closures were very long and openings much less frequent in Q140N and I143N/Y145T channels and the conductance was close to 20% of the main state of WT Kir2.1. The amount of blank tracings from similar time intervals was much higher for Q140N and I143N/Y145T than WT Kir2.1, K117N, S128N and F147N/C149T. The decreased openings (large amount of blank tracings) and reductions in current amplitude of Q140N and I143N/Y145T channels correlated with the observed small whole-cell currents.

To determine whether glycosylated and unglycosylated channels could be differentiated at the cell surface, whole-cell current measurements were made in the absence and presence of tunicamycin for each mutant (Table 1). These measurements are intended to compare the steady state current at –120 mV for each mutant, with and without tunicamycin, and not to compare the mutants to WT Kir2.1. Steady state current measurements at similar post-infection times, in the absence or presence of tunicamycin, were not significantly different (S128N, -297 ± 111 pA/pF, n=3; -275 ± 87 pA/pF, n=4; Q140N, -2.18 ± 0.59 pA/pF, n=5; -4.35 ± 3.5 pA/pF, n=7; I143N/Y145T, -2.78 ± 1.17 pA/pF, n=5; -3.98 ± 2.29
Significance was determined using t-Test of two populations. Typical whole-cell current tracings of tunicamycin-treated infected cells are shown in Figure 3C. Current patterns were similar to untreated infected cells.

**K⁺/Na⁺ Selectivity and Ba²⁺ Block**

Kir2.1 is highly selective for potassium ions and externally applied barium ions block the currents. Mutations and N-linked oligosaccharides introduced in or near the putative pore-forming segment may alter K⁺ selectivity. Ion selectivity was examined by replacing K⁺ with Na⁺ in the extracellular bath solution (Figure 4). Substitution of Na⁺ produced large reductions in the inward currents and hyperpolarizing shifts of the reversal potential for WT Kir2.1 (-64.3 ± 10.9 mV, n=4), K117N (-64.5 ± 4.2 mV, n=4), S128N (-64.0 ± 8.7 mV, n=4) and F147N/C149T (-64.3 ± 7.3 mV, n=4). The experiments show that the reversal potential shifted in the potassium reversal potential direction, which suggests that the N-glycosylation substitution mutants are selective for K⁺ over Na⁺.

Whole-cell currents detected from Sf9 cells expressing WT Kir2.1 and all of the Kir2.1 N-glycosylation substitution mutants were blocked by barium (Figure 5). Barium blockade of Kir2.1 is time- and voltage-dependent (2). The current-voltage (I-V) curve reveals outward currents at more positive pulses near the zero-current potential that become much smaller at more depolarized potentials (Figure 5A, right panel). The negative slope conductance at these potentials is characteristic of strong inwardly rectifying K⁺ channels, Kir2.1. It is less pronounced in the presence of extracellular Ba²⁺ or intracellular spermine when Kir 2.1 is heterologously expressed in *Xenopus* oocytes (4-6) or other cell lines (21, 22). This hump in the outward current was easily detectable when the current magnitudes
were relatively large and it was blocked by barium as shown for S128N and F147N/C149T. The currents for WT, K117N, S128N and F147N/C149T were quite large so barium block is shown in the low K⁺ solution whereas the currents for Q140N and I143N/Y145T were very small so barium block studies were conducted using the high K⁺ solution.

**Detection of Glycosylated Protein from the Plasma Membrane**

Previously, we have shown that glycosylated and unglycosylated Kir1.1 channels could be differentiated at the cell surface by reductions in whole-cell currents and opening probabilities before and after tunicamycin treatment (15-17). Tunicamycin treatment produced no significant changes in the currents for glycosylated Kir2.1 N-glycosylation substitution mutants (S128N, Q140N, I143N/Y145T and F147N/C149T, Table 1). To demonstrate that glycosylated channels are transported to the plasma membrane, surface proteins were biotinylated, and bulk purified using strepavidin-agarose resin. Kir2.1 mutant proteins were detected by Western blotting using the \textit{M2} antibody (Figure 6). Q140N migrated as a singlet. When Q140N samples were digested with endoglycosidase H, a doublet was observed. The lower band was the most prominent band, indicating that the majority of oligosaccharide was removed from glycosylated Q140N protein. I143N/Y145T ran as a doublet and only the lower band was present after digestion with endoglycosidase H. In both cases, the amount of glycosylated protein was higher when glycosylation was assayed from surface expressed Kir2.1 proteins than total Kir2.1 proteins. In fact, virtually all of the Q140N protein that was transported to the cell surface was found to be glycosylated.
Discussion

In this study, we conducted experiments on Kir2.1 channels in which N-glycosylation sites were introduced in and near the putative pore-forming segment, H5. Measurements of partially purified Kir2.1 proteins from total membranes showed a slower migrating immunoband for S128N, Q140N, I143N/Y145T and F147N/C149T that was not present when cells were treated with tunicamycin or in WT Kir2.1 samples. Immunoband shift assays of partially purified Q140N and I143N/Y145T proteins from the plasma membrane also detected a slower migrating band that was substantially reduced when samples were digested with endoglycosidase H. These results indicate that introduced N-glycosylation sequons at positions 128, 140, 143 and 147 could be utilized, and thus provide direct evidence that H5 is extracellular (Figure 1A, right panel). We propose one extracellular loop between the two transmembrane segments because H5 is not in the membrane (Figure 1A, right panel), unlike the accepted topological model that proposes two extracellular loops because H5 is in the membrane (Figure 1A, left panel).

According to the accepted topological model the N-glycosylation sites at position 117 and 128 should be glycosylated and those at position 140, 143 and 147 should not be accessible to the oligosaccharyltransferase (Figure 1A, left panel). However, all three introduced N-glycosylation sequons in the putative pore-forming segment were utilized but residue 117 was not. It may be that residue 117 is not accessible to the oligosaccharyltransferase because it is too close to the M1 segment or the secondary structure does not support the oligosaccharyltransferase reaction (18, 23, 24). Previously, cysteine scanning mutagenesis studies revealed that the side chains of residues 141-143, 145, 147 and 149 are accessible from the extracellular side (25, 26). Thus, two independent approaches demonstrate that residues in H5, including the selectivity filter, are accessible to an
endoplasmic reticulum luminal enzyme and externally applied modifying reagents, indicating that the selectivity filter is extracellular.

In the case of voltage-gated \( K^+ \) channels, \( K_{\text{vs}} \), mutagenesis-electrophysiological and N-glycosylation studies used to evaluate the topological structure are not in agreement. Based on substituted cysteine accessibility method (10), as well as internal tetraethylammonium blockade (7), central residues have been predicted to face the cytoplasmic side, and thus penetrate the membrane. On the other hand, a \( K_v \) channel, Herg, has a utilized native N-glycosylation sequon that directly follows the invariant GY/FG sequence (Figure 1B, 27), indicating that the signature sequence is topologically extracellular. This observation is entirely consistent with our findings for the Kirs, Kir1.1 F146N/F148S, mutant (16) and Kir2.1 F147N/C149T (Figure 1B).

Functional measurements indicated that 117, 128, 140, 143 and 147 mutant channels are at the cell surface. In all cases, whole-cell currents showed strong inward rectification and were blocked by barium. In contrast, the single channel current amplitudes were greatly reduced for Q140N and I143N/Y145T, and opening probabilities were also very low. These different current properties may indicate that the channels are folded incorrectly and therefore glycosylation occurs only when the H5 region is folded abnormally. However, the current amplitude exhibited by these two mutants appear to be similar to the low subconductance state detected in Kir2.1 channels expressed in \textit{Xenopus} oocytes, HEK cells and mouse cardiac myocytes (28), as well as rabbit ventricular myocytes (29). This would suggest that the channels are folding properly but the WT Kir2.1 main open state is not stable. Furthermore, cysteine mutagenesis studies indicated that the H5 segment in the mature protein is accessible from the extracellular side (25, 26). Immunoband shifts by endoglycosidase H showed that virtually all the Q140N protein in the plasma membrane was glycosylated and that a higher
ratio of glycosylated I143N/Y145T was present at the plasma membrane than in total membranes. This result indicates that either more glycosylated protein is transported to the cell surface or that it is more stable. Taken together, the results suggest that glycosylated Q140N channels are correctly folded at the cell surface and functional, as well as the other glycosylated channels, S128N, I143N/Y145T and F147N/C149T.

A previous study of Kir2.1 reported that R148 and E138 form a salt bridge, and that disruption of this bridge dramatically alters ion selectivity and permeation (30). These observations are further supported by the Kir6 model, which was built from the crystal structure of KcsA (31). However, a more recent study has demonstrated that K⁺ selectivity is maintained when a Met is substituted at R148 in Kir2.1 but permeation properties are changed (32). Interestingly, our study shows that nonconserved mutations on each side of R148 do not abolish K⁺ selectivity but the current amplitudes are reduced by about 55% (see Figure 3). K⁺ selectivity was also retained when the two corresponding residues were mutated in Kir1.1 (16). The double mutants also had N-linked oligosaccharide at positions 147 and 146 of Kir2.1 and Kir1.1, respectively. Taken together, this region appears to be either directly involved or tightly coupled to ion permeation, not ion selectivity.

Our studies clearly demonstrate that introduced N-glycosylation sites in the putative pore-forming segment can be utilized and that the mutant channels retain function. At least two plausible explanations could explain glycosylation of these introduced sites. First, the selectivity filter is extracellular, and thus ion selectivity originates from an extracellular segment. Alternatively, ion selectivity does not originate from this segment. Mutagenesis-electrophysiological studies cannot clearly establish whether ion selectivity, ion permeation and channel gating were altered as a result of those specific amino acids and modifications, or were the result of allosteric effects. In other words, mutations or modifications outside the
pore are coupled to the pore structure, and thus alter ion channel properties. It is possible that protein-protein contacts of the multimeric protein channel may affect the highly positive cooperativity of the transition between closed and main open states. Thus, disrupting protein-protein contacts of the multimeric protein channel would alter channel gating, ion permeation and/or ion selectivity.

In conclusion, our study of a strong inward rectifier, and our earlier study on a weak inward rectifier (15-17) show that H5, the putative pore-forming segment, of Kir2 is an extracellular segment because positions 128, 140, 143 and 147 of Kir2.1 and positions 117, 128, 139, 144 and 146 of Kir1.1 are glycosylated. Positions 139, 140, 143, 144, 146 and 147 reside in the signature sequence that is thought to form the selectivity filter. Occupancy of N-glycosylation sequons at these sites places the selectivity filter of the signature sequence in an extracellular loop as depicted in Figure 1A (right panel), rather than within the membrane (Figure 1, left panel). We speculate that the region containing the invariant GY/FG sequence is topologically extracellular for all K⁺ channels.
References


Footnotes
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1 Abbreviations used: Kir, inwardly rectifying K+ channel; WT, wildtype, Kvs, voltage-gated K+ channels; Sf9, Spodoptera frugiperda; MES, 2-(N-morpholino)ethanesulfonic acid; M1, 1st transmembrane segment; M2, 2nd transmembrane segment; E1, 1st extracellular loop; E2, 2nd extracellular loop; H5, putative pore-forming segment; M2, a peptide (DYKDDDDK).

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Figure Legends

Figure 1. Topological structures of Kir2.1 and amino acid sequences of the putative pore-forming segment of various K⁺ channels. A, the accepted topological model of Kir2.1 consists of 428 amino acid residues (left panel). Hydropathy plots predict two membrane-spanning segments (M1 and M2) and two extracellular loops (E1 and E2) which link a putative pore-forming segment (H5). Cytoplasmic N- and C-termini are based on the absence of signal sequence. A, our biochemically derived topological model based on N-glycosylation tagging (right panel). Orientation of M1, M2, N- and C-termini are similar to the accepted topological model. The difference is the presence of one extracellular loop that consists of the putative pore-forming segment (H5) and two putative extracellular loops (E1 and E2). The number adjacent to each dot is the Asn residue, representing the position of each introduced N-glycosylation sequon (NXS/T). The branched structure attached to the dots denotes N-linked oligosaccharide. GYG represents the invariant tripeptide sequence (GY/FG) in H5. B, The amino acid sequence of the putative pore-forming segment (H5) of Kir2.1, a strong inwardly rectifying potassium channel, Kir1.1, a weak inwardly rectifying potassium channel, two voltage-dependent potassium channels, Herg and Kv3.1, and a bacterial potassium channel, KcsA. Eight consecutive residues underlined represent the signature sequence of K⁺ channels. Underlined individual letters indicate the first amino acid residue, Asn, of the tripeptide signal where glycosylation was detected. Two lightened G residues are part of the invariant GY/FG sequence of K⁺ channels.

Figure 2. Immunoblot analysis of Kir2.1 constructs. Sf9 cells were infected with recombinant baculovirus, encoding WT Kir2.1 or Kir2.1 N-glycosylation mutant cDNA, and subsequently infected cells were treated without (-) or with (+) tunicamycin (25 µg/ml). TM
is the abbreviation of tunicamycin. Proteins were immunoaffinity purified from total membranes as described in ‘Materials and Methods’. Partially purified samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane for immunoblotting with anti-M2 antibody. Arrows represent positions of glycosylated (upper) and unglycosylated (lower) forms of Kir2.1.

**Figure 3. Functional expression of WT Kir2.1 and Kir2.1 N-glycosylation substitution mutants.** *A, Left column,* whole cell currents of Sf9 cells expressing WT Kir2.1 and Kir2.1 N-glycosylation substitution mutants were generated by either 200 ms (WT, K117N, Q140N and F147N/C149T) or 250 ms (S128N and I143N/Y145T) pulses from -120 mV to +80 mV in 20 mV increments at a holding potential of 0 mV, as indicated. *Right column,* representative single channel recordings of the same infected cell expressing WT Kir2.1, K117N, S128N, Q140N, I143N/Y145T or F147N/C149T. Single channel currents in the cell-attached mode were recorded for 10 s at -100 mV from a holding potential of 0 mV. All representative single channel traces are 200 ms. *B, control* is a whole-cell record from a cell infected with a recombinant baculovirus encoding the cytoplasmic N-terminus segment of H, K-ATPase α2A (residues 1-110). The following three records are representatives of cells expressing Q140N, Y143N/Y145T and F147N/C149TN with similar amounts of leak current. *C, typical* whole-cell current recordings of infected cells treated with tunicamycin. Currents were recorded in a high K⁺ solution containing (in mM): K-aspartate 140, MgCl₂ 1, MES 10, Mannitol 60 (pH 6.3, adjusted with Tris-OH). The pipette solution contained (in mM): K-aspartate 140, MgCl₂ 5, HEPES 10, EGTA 10, Mannitol 40 (pH adjusted to 7.2 with Tris-OH).
Figure 4. N-Glycosylation substitution mutants retain K⁺ selectivity. Currents were elicited by voltage steps from -120 to +80 mV in 20 mV increments at a holding potential of 0 mV in the high K⁺ solution (left side). Cells were then perfused with a low K⁺ solution containing (middle, in mM): Na-aspartate 135, K-aspartate 5, MgCl₂ 1, MES 10, Mannitol 60 (pH 6.3, adjusted with Tris-OH). The current-voltage (I-V) relationships show a large negative shift in the reversal potential when perfusing cells from a high K⁺ solution (circles) to a low K⁺ solution (triangles).

Figure 5. Barium Blockade of WT Kir2.1 and Kir2.1 N-glycosylation mutants. A, whole-cell currents of WT Kir2.1 and N-glycosylation mutants in a low K⁺ solution, minus and plus 7 mM BaCl₂, were elicited by voltage steps from –120 to +80 mV in 20 mV increments from a holding potential of –60 mV. Current-voltage relationships of WT and mutants are shown on the right panel. Squares and circles are without and with BaCl₂, respectively. B, currents for Q140N and I143N/Y145T were small, unlike WT Kir2.1 and the above mutants, so barium blockade were conducted in a high K⁺ solution, minus and plus 7 mM BaCl₂. A similar voltage step protocol was used, except the holding potential was 0 mV.

Figure 6. Glycosylated Kir2.1 proteins detected at the cell surface. Surface proteins from infected Sf9 cells expressing Q140N and I143N/Y145T were biotinylated, and subsequently purified as described in ‘Materials and Methods’. Partially purified biotin-labeled protein samples were digested without (-) and with (+) endoglycosidase H (EndoH). Samples were separated on 12% SDS gels and probed with the M2 antibody. Upper and lower arrowheads represent glyco- and aglyco-forms, respectively.
Table 1. Steady-state currents at –120 mV in the absence and presence of tunicamycin.

<table>
<thead>
<tr>
<th>Kir2.1:</th>
<th>S128N</th>
<th>Q140N</th>
<th>I143N/Y145T</th>
<th>F147N/C149T</th>
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<tbody>
<tr>
<td>TM:</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>I_{ss}/cap (pA/pF)</td>
<td>297±111</td>
<td>-275±87</td>
<td>-2.18±0.6</td>
<td>-1.78±7.4</td>
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<td>111</td>
<td>-275</td>
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The steady-state currents (I_{ss}) at –120 mV were determined and divided by the cell capacitance (cap) in the absence (-) and presence (+) of tunicamycin (TM) for each N-glycosylation substitution mutant, as indicated. The number of observations is indicated in the corresponding n columns.
B.

H5- or P-loop

Kir2.1  AAFLESIETQTTIGYGFRC
Kir1.1  SAFLFSLETQVTIGYGFRC
Herg    TALYFTFSSLTSVGFGNVS
Kv3.1   IGFWAATMTLGYGDML
KcsA    RALWMSVETATTVGYGDLY

Pore Signature Sequence
Site-directed glycosylation tagging of functional Kir2.1 reveals that the putative pore-forming segment is extracellular
Ruth A. Schwalbe, Alicia Rudin, Shen-Ling Xia and Charles S. Wingo

J. Biol. Chem. published online May 3, 2002

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