Glycosylation of Shaker Potassium Channel Protein in Insect Cell Culture and in *Xenopus* Oocytes†

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ABSTRACT: We have studied the glycosylation of Shaker K^+ channel protein made in two expression systems: an insect cell culture line and amphibian oocytes. In both systems, two potential sites for N-linked glycosylation were modified. The modified sites were located between the first and second putative transmembrane segments, S1 and S2. Although the same sites appeared to be glycosylated in both systems, the fraction of protein glycosylated and the size, structure, or composition of the oligosaccharide chains added were quite different. The results indicate that the S1–S2 loop is extracellular, consistent with a cytoplasmic location for the N-terminus and a transmembrane disposition for hydrophobic segment S1. We have also shown that glycosylation occurs in two stages in oocytes, generating an immature and a mature form of Shaker protein. However, glycosylation is not required either for the assembly of functional channels or for their transport to the cell surface.

Potassium channels are integral membrane proteins that control the permeability of cells to K^+ and contribute to the excitability of nerve and muscle (Hille, 1991). The cloning of genes for voltage-dependent K^+ channels has led to structure/function analysis through a combination of site-directed mutagenesis and electrophysiology (Miller, 1991). Because electrophysiological methods provide extremely sensitive measurements of channel function, including the molecular transitions of single molecules (Hamill et al., 1981), much has been learned about the contributions of different regions of the sequence to the various functional properties of K^+ channels (Jan & Jan, 1992). However, detailed structural information will be needed to achieve a more complete understanding of channel mechanisms.

At present, little is known about the biochemical nature of K^+ channels. Many K^+ channels are part of a superfamily that also includes voltage-dependent Na^+ and Ca^{2+} channels (Jan & Jan, 1988). Each member of this family has a similar pattern of hydrophobic segments in its subunits or pseudo-subunits, which has led to the idea that these domains assume a similar topology in the membrane. K^+ channels are likely to be composed of four subunits that form the ion-conducting structure (MacKinnon, 1991). In expression systems and in neurons, K^+ channels may be formed by identical or nonidentical subunits (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; Sheng et al., 1993; Wang et al., 1993). Models for the topological disposition of K^+ channel subunits in the membrane have been inferred from hydrophobicity analysis and the functional effects of site-directed mutations (Miller, 1991).

The Shaker gene of *Drosophila*, which encodes a voltage-dependent, inactivating K^+ channel, has been expressed in several heterologous systems for electrophysiological analysis (Timpe et al., 1988a,b; Leonard et al., 1989; Klaiber et al., 1990; Choi et al., 1991). We are studying the biochemical properties of Shaker K^+ channels in some of these expression systems, including the baculovirus system and *Xenopus* oocytes. An insect cell line (Sf9) infected with a recombinant baculovirus containing the Shaker gene produces functional K^+ channels and a large amount of Shaker protein (Klaiber et al., 1990). It is not known what fraction of the protein made by the baculovirus system can be assembled into active channels, since much of it may not be transported to the plasma membrane (Klaiber et al., 1990; John and Papazian, unpublished observations). In this insect-based system, however, the biochemical processing of the protein may resemble that which occurs in its natural environment in cells of the fruit fly. The system of choice for studying the functional effects of site-directed mutations has been oocytes of the frog, *Xenopus laevis* (Miller, 1991). Previously, it has been reported that some heterologous membrane proteins are modified differently in *Xenopus* oocytes than in their native environment (Buller & White, 1990).

We have characterized the glycosylation of Shaker protein made in Sf9 cells infected with the Shaker recombinant baculovirus and in *Xenopus* oocytes injected with Shaker RNA. In both systems, the protein is glycosylated at two of four consensus sites. The modifications occur at two asparagines in the loop connecting the first and second putative membrane-spanning segments, consistent with an extracellular location for this loop. Although the same sites appear to be modified, the size, structure, or composition of the oligosaccharide chains attached in oocytes is different than in the insect system. In addition, we have shown that glycosylation occurs in two stages in oocytes, resulting in an immature and a mature form of the protein. Glycosylation is not required for the transport of active channels to the oocyte surface, and the carbohydrate moieties do not appear to modify the functional properties of the channel.

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MATERIALS AND METHODS

Antisera. Antisera (kindly provided by L. Y. Jan and Y. N. Jan, University of California, San Francisco) were raised in rabbits by Berkeley Antibody Co. (Richmond, CA) against synthetic peptides conjugated to keyhole limpet hemocyanin. Anti-NH_2-terminal antiserum was directed against the peptide NH_2-Cys-His-Gln-Lys-Glu-Gln-Leu-Glu-Gln-Lys-Glu-Glu-Gln-Lys-Lys-Ile-Ala-Glu-Arg-Lys-COOH, which represents amino acids 26–44 of the Shaker H4 sequence (Kamb et al., 1988), plus an N-terminal cysteine for coupling to carrier protein. Anti-S1–S2 loop antiserum was directed against the peptide NH_2-Cys-Thr-Thr-Thr-Asn-Gly-Thr-Lys-Ile-Glu-Glu-Asp-Glu-Val-Pro-Asp-Ile-Thr-Asp-Pro-COOH, which represents amino acids 260–278 of the Shaker H4 sequence (Kamb et al., 1988), plus a cysteine for coupling. Antisera against a Shaker–β-galactosidase fusion protein and a peptide representing the S3–S4 loop have been described previously (Schwarz et al., 1990).

Sf9 Cell Culture and Preparation of Crude Membrane Fraction. Sf9 cells were infected at a multiplicity of 5–10 with a wild-type baculovirus or a recombinant baculovirus encoding the Shaker H4 protein (Klaiber et al., 1990). At 24-h postinfection, a mixed membrane fraction was prepared as described (Klaiber et al., 1990), except that cell pellets were resuspended in 10 mM sodium phosphate/1 mM EDTA, pH 7.0, and the membrane fraction was resuspended in 150 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 10 mM sodium phosphate, pH 7.2. Solutions were supplemented with protease inhibitors (obtained from Sigma, Saint Louis, MO, or Boehringer Mannheim, Indianapolis, IN) as follows: 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 50 μg/mL antipain, 25 μg/mL (4-amidinophenyl)methanesulfonyl fluoride, 40 μg/mL bestatin, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.1 mM EDTA. Where indicated, cells were infected and grown in media containing 50 μg/mL tunicamycin (Sigma).

Molecular Biology and Expression of Channels in Xenopus Oocytes for Voltage Clamp Analysis. The Shaker B cDNA or mutants derived from it were used in oocyte experiments. The protein encoded by Shaker B is identical to that encoded by Shaker H4 with the exception of two amino acids, and it contains the same consensus sites for N-linked glycosylation (Tempel et al., 1987; Kamb et al., 1988; Schwarz et al., 1988). The two amino acid differences between Shaker H4 and Shaker B are at positions 513 and 558, and are not in regions that affect the results we report. Mutations were generated by oligonucleotide-directed mutagenesis using the dut$^-$ung$^-$ genetic selection (Kunkel, 1987; Sambrook et al., 1989). They were confirmed by complete sequencing of the mutagenized region through the nearest unique restriction sites, which were used to transfer the mutation into the complete Shaker construct. RNA was prepared, and 1–5 ng was injected into defolliculated *Xenopus laevis* oocytes (Timpe et al., 1988a,b; Papazian et al., 1991). Oocytes were assayed for the functional expression of Shaker K^+ channels with a two-electrode voltage clamp (Warner Electronics). Activation–voltage and steady-state inactivation–voltage relationships were determined as previously described (Timpe et al., 1988a). Voltage pulses were delivered and data were collected using a 80386 computer and pClamp v.5.5.1 software (Axon Instruments).

Biochemical Analysis of Shaker Protein Made in Oocytes. Oocytes were injected with 25–40 ng of RNA made from a construct that contained a deletion of amino acids 6–46 (Shaker-IR). This deletion removes fast inactivation (Hoshi et al., 1990) and appears to increase the level of protein expression compared to the wild-type construct (unpublished observations). Shaker protein was metabolically labeled by continuously incubating the oocytes in ND-96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5, supplemented with 0.55 mg/mL sodium pyruvate, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 5 μg/mL gentamycin) supplemented with 25 μCi/mL of a mixture of [^{35}S]methionine and [^{35}S]cysteine (Translabel, ICN) or by injecting 400 nCi/oocyte of in vitro translation-grade [^{35}S]-methionine (ICN) followed by incubation in ND-96 lacking radiolabel. At various times after injection, oocytes were suspended in 10% sucrose (w/v) in 150 mM NaCl, 5 mM KCl, 10 mM magnesium acetate, and 20 mM HEPES, pH 7.5, supplemented with protease inhibitors as described above, and briefly sonicated. The homogenate was overlaid on a 10–20–50% discontinuous sucrose gradient prepared in the same buffer. Gradients were centrifuged at 170000*g* for 30 min at 15 °C. A membrane fraction was collected from the 20–50% sucrose interface, diluted at least 2.5-fold with cold water, and pelleted by centrifugation at 170000*g* for 10 min at 4 °C. Membrane proteins were solubilized in 75 mM KCl, 75 mM NaCl, and 50 mM sodium phosphate (pH 7.2), containing 2 mg/mL crude soybean lipids (Avanti) and 2% Lubrol-PX (w/v) (Sigma), supplemented with protease inhibitors. Insoluble material was removed by centrifugation at 100000*g* for 30 min at 4 °C. Shaker protein was immunoprecipitated using an antiserum to a Shaker–β-galactosidase fusion protein or an anti-peptide antiserum against the putative extracellular loop between S3 and S4 (Tempel et al., 1987; Schwarz et al., 1990), and protein A–Sepharose beads (Sigma). The beads were pelleted in a microcentrifuge and extensively washed with 1% Triton X-100 (w/v), 100 mM NaCl, 100 mM Tris (pH 8.0), and 10 mM EDTA (pH 8.0), supplemented with 2 mM phenylmethanesulfonyl fluoride. Beads were resuspended in Laemmli sample buffer containing 10% 2-mercaptoethanol and boiled for 3 min. Protein samples were stored at –80 °C until they were subjected to electrophoresis as described below. Each lane contained material extracted from approximately 20 oocytes. Gels were dried and subjected to autoradiography for 5 days to 2 weeks.

Enzymatic Deglycosylation. The Sf9 cell membrane fraction was resuspended at a protein concentration of 0.7–0.8 mg/mL in 20 mM potassium phosphate/50 mM EDTA, pH 7.4, plus protease inhibitors. SDS and 2-mercaptoethanol were each added to a final concentration of 1% (w/v and v/v, respectively, and the suspension was boiled for 3 min. A 3-fold excess by weight of Nonidet P-40 over SDS was added, and the sample was diluted with an equal volume of 20 mM potassium phosphate/50 mM EDTA, pH 7.4, plus protease inhibitors. Five units of *N*-glycosidase F (Genzyme, Cambridge, MA) were added to a 20-μL aliquot, and the sample was incubated for 16 h at 37 °C.

Oocyte protein samples were enzymatically deglycosylated by one of two procedures. After immunoprecipitation and washing of Shaker protein using protein A–Sepharose beads, the beads were subjected to additional washes with 20 mM potassium phosphate/50 mM EDTA, pH 7.4. Beads were then resuspended in 35 μL of the same buffer, supplemented with SDS and 2-mercaptoethanol to final concentrations of 1% (w/v) and 1% (v/v), respectively, and boiled for 3 min. Nonidet P-40 was added to a final concentration of 3% (w/v). The sample was diluted with an equal volume of buffer and digested with 5 units of enzyme for 16 h at 37 °C.

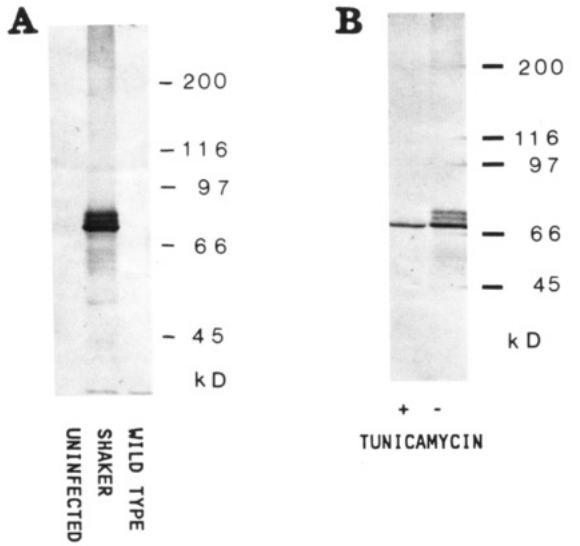

FIGURE 1: Shaker protein produced in Sf9 cells is glycosylated. Immunoblots were probed with an antiserum against a Shaker–β-galactosidase fusion protein (Schwarz et al., 1988). (A) shows an immunoblot of crude membrane fractions from uninfected Sf9 cells, or cells infected with a Shaker recombinant baculovirus, or a wild-type baculovirus as indicated. (B) shows an immunoblot of membranes from Sf9 cells infected with the Shaker recombinant baculovirus and treated with tunicamycin (50 μg/mL), as indicated. The following proteins were used as molecular mass standards: myosin (200 000 daltons), β-galactosidase (116 250 daltons), phosphorylase *b* (97 400 daltons), bovine serum albumin (66 200 daltons), and ovalbumin (45 000 daltons).

Alternatively, the buffer used for washes and the digest was replaced by 100 mM NaCl, 50 mM Tris-HCl, and 1 mM $CaCl_2$, pH 8. Beads were incubated in 25 μL of buffer and digested with 1.9 units of *N*-glycosidase F for 16 h at 20 °C. The reaction was terminated by addition of 12.5 μL of sample buffer, and boiled as above.

Electrophoretic Analysis, Immunoblots, and Protein Assays. Samples were diluted with an equal volume of Laemmli sample buffer containing 10% 2-mercaptoethanol and were subjected to electrophoresis on 7.5% acrylamide separating gels with 4% stacking gels. Immunoblots were performed as previously described (Schwarz et al., 1990). Primary antisera were diluted 250-fold before use on immunoblots. Alkaline phosphatase-conjugated secondary antibody was purchased from Capell (Organon Teknika Corp., Durham, NC) and diluted 7500-fold before use. Immunoblots were developed using the chromogenic reagents nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Protein assays were performed as described (Smith et al., 1985).

RESULTS

Glycosylation of Shaker Protein in the Baculovirus Expression System. Sf9 cells infected with a recombinant baculovirus encoding the Shaker H4 K^+ channel produce a large amount of immunoreactive Shaker protein (Klaiber et al., 1990). Membrane fractions were prepared from uninfected Sf9 cells, or from cells infected with the recombinant baculovirus, or a wild-type baculovirus. The membrane fractions were analyzed on immunoblots probed with an antiserum against a Shaker–β-galactosidase fusion protein (Schwarz et al., 1990). Cells infected with the Shaker recombinant virus expressed a triplet of immunoreactive proteins with relative molecular masses of 71 000, 74 000, and 77 000 daltons (Figure 1A). [The deduced amino acid sequence of the Shaker H4 protein predicts a molecular mass of 74 000 daltons (Kamb et al., 1988).] The triplet of proteins was not present in uninfected cells or in cells infected with a wild-type baculovirus.

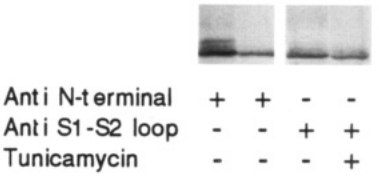

FIGURE 2: Reactivity of Shaker protein with anti-peptide antibodies. Immunoblots of membrane fractions were probed with antisera raised against peptides from the N-terminal region or the interval between S1 and S2. S1 and S2 are the first two of six segments identified by hydrophobicity analysis as potential transmembrane sequences (Tempel et al., 1987). The interval between S1 and S2 contains two asparagine residues that are potential sites for N-linked carbohydrate addition [consensus sequence Asn-X-Thr/Ser (Hubbard & Ivatt, 1983)]. They are Asn-259, which is the amino acid in the protein immediately preceding the synthetic peptide used to raise anti-S1–S2, and Asn-263, which is included in the synthetic peptide. Cells were treated with 50 μg/mL tunicamycin as indicated. Molecular mass standards are as in Figure 1.

Cells infected with the Shaker recombinant virus were treated with tunicamycin, an inhibitor of N-linked glycosylation. Immunoblots revealed that these cells contained a single Shaker band that comigrated with the smallest membrane of the triplet of immunoreactive proteins present in untreated cells (Figure 1B). Similarly, treatment of membrane fractions from Shaker-infected cells with *N*-glycosidase F (*N*-glycanase), which removes N-linked carbohydrate from proteins, converted the triplet to a single band that comigrated with the smallest member of the triplet and the single band made in tunicamycin-treated cells (data not shown). These results indicate that the Sf9 cells produce three forms of Shaker protein. The lowest band of the triplet is not glycosylated, whereas the two upper bands represent protein differing in the extent of glycosylation.

The Shaker H4 protein sequence contains four potential sites for N-linked glycosylation (Tempel et al., 1987; Kamb et al., 1988; Schwarz et al., 1988). One site (Asn-102) is in the N-terminal region, two (Asn-259 and Asn-263) are in an interval between S1 and S2, the first two of six potential transmembrane segments identified by hydrophobicity analysis, and one (Asn-625) is in the C-terminal region (Schwarz et al., 1988). To attempt to localize the sites of carbohydrate attachment to the Shaker protein in Sf9 cells, immunoblots were probed with anti-peptide antibodies. An antiserum raised against a synthetic peptide representing the S1–S2 loop preferentially recognized the unglycosylated, 71 000-dalton member of the triplet on immunoblots (Figure 2). This antiserum recognized the same pattern in membrane proteins from tunicamycin-treated cells. In contrast, an antiserum directed against an N-terminal peptide detected the triplet of bands in membranes from untreated cells and one band after tunicamycin treatment. Although the two anti-peptide antibodies detected the unglycosylated Shaker protein with approximately the same intensity, only the anti-N-terminal antiserum strongly detected the glycosylated forms of the protein. These results suggest that carbohydrate addition interferes with the ability of the antibody directed against the S1–S2 loop to recognize its peptide epitope and that Shaker protein made in Sf9 cells may be glycosylated at either one or two sites in the S1–S2 loop, accounting for the two upper bands of the immunoreactive triplet.

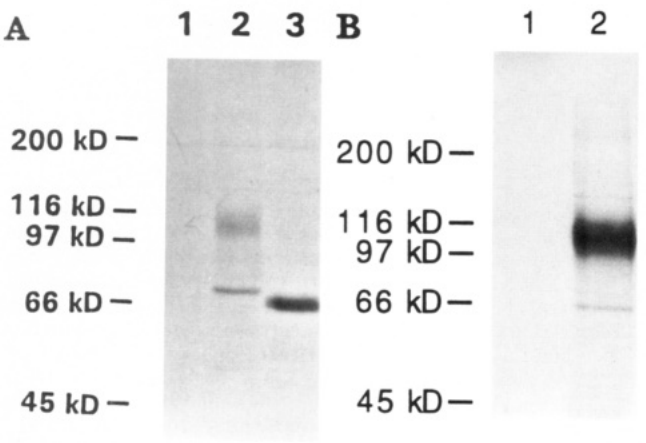

FIGURE 3: Oocytes make two glycosylated forms of the Shaker protein. Autoradiogram of samples immunoprecipitated from solubilized crude membrane fractions. (A) Oocytes were metabolically labeled by incubation in ND-96 supplemented with 25 μCi/mL of a mixture of [^{35}S]methionine and [^{35}S]cysteine. Shaker protein was immunoprecipitated with an antiserum against a Shaker–β-galactosidase fusion protein. Lane 1, control; oocytes were injected with water only. Lane 2, oocytes were injected with RNA coding for the Shaker protein. Lane 3, immunoprecipitated protein was enzymatically deglycosylated with *N*-glycosidase F. (B) Oocytes were labeled by co-injection of RNA and 400 nCi of in vitro translation-grade [^{35}S]methionine. Shaker protein was immunoprecipitated with an anti-peptide antibody directed against the putative extracellular loop between S3 and S4. This epitope does not overlap the region of Shaker sequence present in the β-galactosidase fusion protein. Molecular mass standards are as in Figure 1.

Biochemical Characterization of Shaker Protein Made in Oocytes. To characterize the sites of carbohydrate addition more thoroughly, we studied the glycosylation of Shaker protein made in *Xenopus* oocytes. Biochemical characterization of the Shaker channel in oocytes is of particular interest since this has been the system of choice for characterizing the functional effects of site-directed mutations (Miller, 1991). Shaker protein made in oocytes from the Shaker-IR construct was metabolically labeled, solubilized, immunoprecipitated, and detected by autoradiography. The Shaker-IR construct contains a deletion of amino acids 6–46, which removes inactivation and appears to increase the level of protein expression compared to the wild-type construct. Two protein bands were immunoprecipitated with anti-Shaker antibodies (Figure 3). One migrated as a sharp band of about 73 000 daltons, whereas the other (which constituted most of the protein) migrated as a very broad band of about 113 000 daltons. We have confirmed that both of these bands are products of Shaker expression because (1) they were not present in oocytes that were metabolically labeled with [^{35}S]-methionine and injected with water instead of RNA (Figure 3A) and (2) they both were specifically immunoprecipitated by antibodies that recognize separate epitopes on the Shaker protein (Figure 3A,B). Both bands contained N-linked carbohydrate: treatment with *N*-glycosidase F converted them to one sharp band of about 68 000 daltons (Figure 3A). The deduced amino acid sequence of the Shaker-IR protein predicts a molecular mass of 69 000 daltons.

The smaller glycosylated protein of 73 000 daltons declines in amount with time after RNA injection (Figure 4). At 24-h postinjection, both bands were detected. At 48-h postinjection, the smaller band had faded, whereas the larger band was more intense. By 72-h postinjection, the smaller band could not be detected. The smaller band may be a precursor of the completely glycosylated protein of about 113 000 daltons, or it may be an unstable form of the protein that is destined for degradation. Because of its incomplete posttranslational modification, this smaller band may represent an immature form of the protein containing only core glycosylation (Rowling & Freedman, 1993).

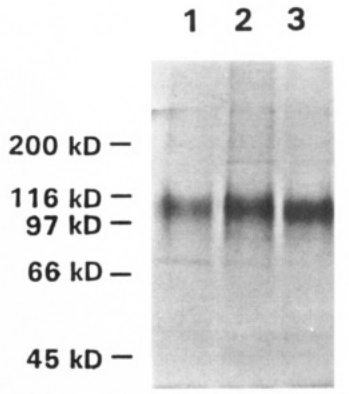

FIGURE 4: Maturation of the Shaker protein in oocytes. Autoradiogram of Shaker protein immunoprecipitated from oocytes at different times after injection. Oocytes were metabolically labeled by injection of 400 nCi of in vitro translation-grade [^{35}S]methionine per cell. Lane 1, 24 h after injection. Lane 2, 48 h after injection. Lane 3, 72 h after injection. Molecular mass standards are as in Figure 1.

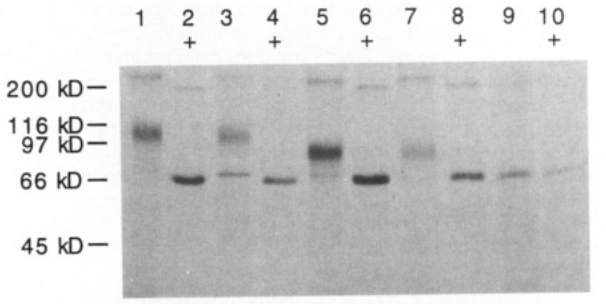

FIGURE 5: Biochemical analysis of the mutants N102Q, N259Q, and N263Q and the double mutant N259Q + N263Q in *Xenopus* oocytes. Autoradiogram of Shaker protein immunoprecipitated from oocytes injected with RNA encoding control protein (Shaker-IR) or glycosylation mutant protein in a Shaker-IR background. Oocytes were metabolically labeled by injection of 400 nCi of in vitro translation-grade [^{35}S]methionine per cell. Samples in lanes marked (+) were enzymatically deglycosylated using *N*-glycosidase F. Lanes 1 and 2, Shaker-IR. Lanes 3 and 4, N102Q-IR. Lanes 5 and 6, N259Q-IR. Lanes 7 and 8, N263Q-IR. Lanes 9 and 10, N259Q+N263Q-IR. Molecular mass standards are as in Figure 1. The upper band present in each lane appears to represent aggregated forms of the Shaker protein.

Localization of Glycosylated Sites. To identify the sites of carbohydrate attachment to the K^+ channel protein in oocytes, we used site-directed mutagenesis of the Shaker gene and biochemical analysis.

Three of the four consensus sites for N-linked glycosylation were individually eliminated by replacing the asparagine in the recognition sequence with glutamine. The protein products of the glycosylation mutants N102Q-IR, N259Q-IR, and N263Q-IR and a double glycosylation mutant, N259Q + N263Q-IR, were compared with Shaker-IR protein at 48 h after injection. The mutations N259Q and N263Q reduced the apparent molecular mass of the IR protein to about 86 000 daltons, that is, to a value about midway between the normal immature and mature forms (Figure 5). In contrast, the protein made by N102Q-IR comigated with that made by Shaker-IR, and therefore appeared to be normally glycosylated. After treatment with *N*-glycosidase F, the protein products of Shaker-IR, N102Q-IR, N259Q-IR, and N263Q-IR were each converted to a sharp band with an apparent molecular mass of about 64 000 daltons. The double glyco-

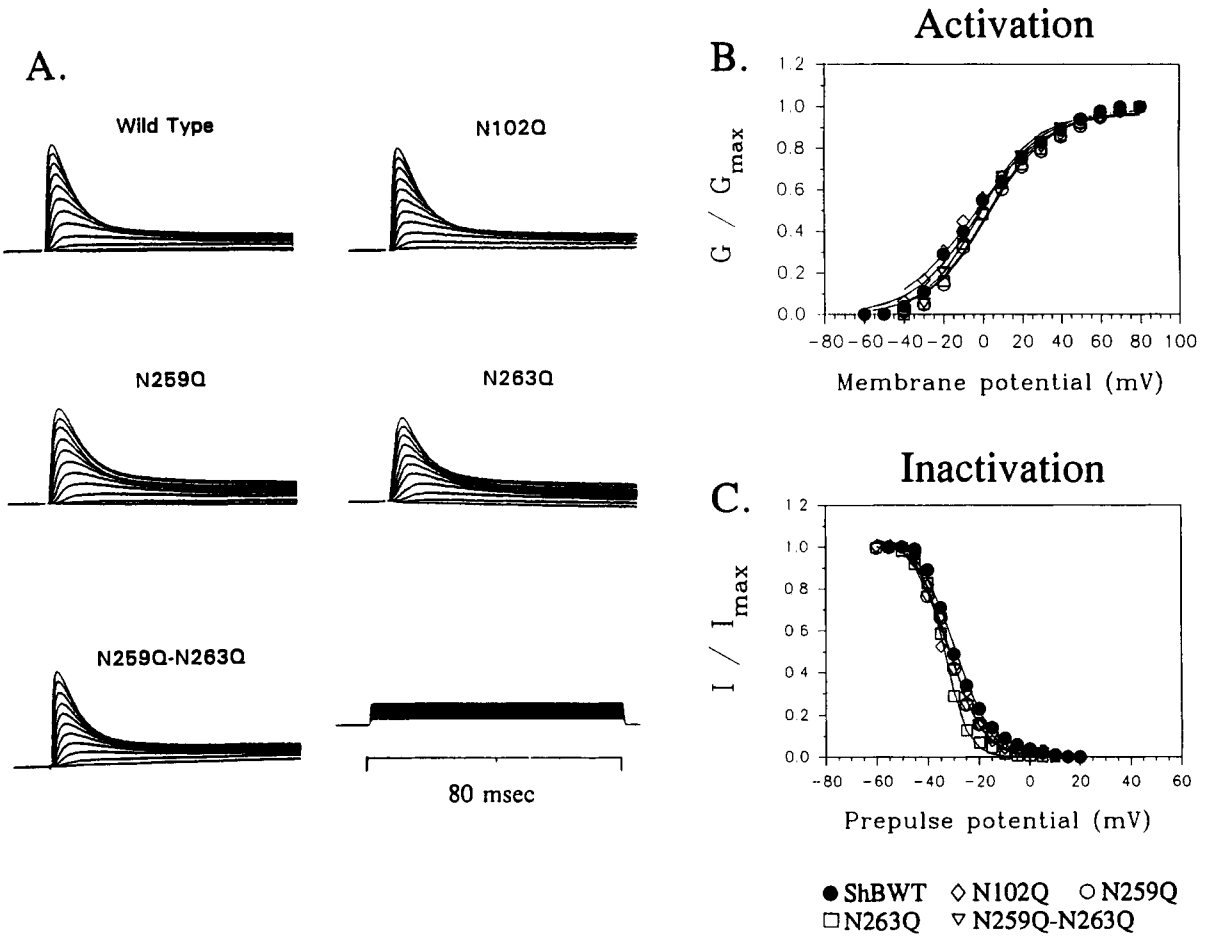

FIGURE 6: Outward potassium currents through the wild-type Shaker channel and glycosylation mutant channels after expression in *Xenopus* oocytes. (A) Transient currents, recorded at 12 ± 1 °C using a two-electrode voltage clamp from wild-type or glycosylation-site mutant channels, were elicited by 80-ms depolarizations from a holding potential of −80 mV to voltages between −40 and +60 mV in 10-mV increments. A linear leakage current was subtracted using the P/−4 protocol (Benzanilla & Armstrong, 1977). Representative peak current amplitudes at +60 mV for the wild-type, N102Q, N259Q, N263Q, and N259Q + N263Q currents shown are 8.2, 8.5, 6.3, 5.7, and 7.4 μA, respectively. Recordings were made from oocytes 36–48 h after RNA injection. (B) The conductance of the channel was calculated by dividing the leak-subtracted peak current by the driving force across the membrane $[I_{peak}/(V_{step} - V_{reversal})]$ (Hille, 1991). $V_{reversal}$ was assumed to be −95 mV under the recording conditions used, 1 mM external K^+. Conductance was normalized to the maximal value, obtained at +80 mV, and plotted as a function of voltage. The solid curves represent the best fit to the Boltzmann equation, $G/G_{max} = \{1 + \exp[(V_{1/2} - V_h)/Z]\}^{-1}$, where $V_{1/2}$ is the midpoint for channel activation, V_h is the holding potential, and Z is the slope factor (Hille, 1991). The mean $V_{1/2}$ values for wild type, N102Q, N259Q, N263Q, and N259Q–N263Q are (in mV, $n = 3$) −1.6, −3.6, 3.1, 2.4, and 3.6, respectively. (C) To characterize the steady-state inactivation properties of wild-type and mutant channels, depolarizing prepulses (400 ms) to various potentials were applied. Then, the fraction of channels inactivated by the prepulse was determined by a subsequent test pulse (50 ms). The peak current during the test pulse was normalized to the maximal peak current measured by depolarizing from a holding potential of −80 mV to the test potential, +60 mV. A noninactivating component of the current was subtracted, and the adjusted, normalized current was plotted as a function of the prepulse potential. The solid curves are the best fit of the data to the Boltzmann equation: $I/I_{max} = \{1 - \exp[(V_{1/2} - V_h)/Z]\}^{-1}$. The mean $V_{1/2}$ values for wild type, N102Q, N259Q, N263Q, and N259Q–N263Q are (in mV) −30.1, −32.7, −32, −33.6, and −31.9, respectively. The symbols display averaged data from three different recordings.

sylation mutant N259Q + N263Q-IR made a protein of about 64 000 daltons that contained no N-linked oligosaccharide, by the criterion that the migration of this protein was unaltered by treatment with *N*-glycosidase F (Figure 5). These results show that N259 and N263 are both modified by glycosylation in oocytes, and that these two sites appear to account for all the N-linked carbohydrate on the protein.

Functional Analysis of the Glycosylation Mutants. To study the functional role of carbohydrate modification of the protein, the glycosylation mutants were characterized using a two-electrode voltage clamp (Timpe et al., 1988a,b). For electrophysiological experiments, the glycosylation mutations were studied in otherwise wild-type constructs that produced inactivating channels. All four mutants generated functional channels that conducted normal-looking K^+ currents (Figure 6A). Furthermore, the voltage dependence of activation and that of steady-state inactivation were not significantly different from those of the wild-type channel (Figure 6B,C). Thus, addition of carbohydrate to the Shaker protein does not appear to modulate the functional properties of the K^+ channel in oocytes. In contrast, deglycosylation of Na^+ channels causes large shifts in the voltage dependence of activation. This effect has been attributed to the presence of negatively-charged sialic acid residues that alter the effective electric field detected by the channel's voltage sensor (Recio-Pinto et al., 1990). Shaker protein produced in oocytes does not appear to contain significant amounts of sialic acid since the migration of the protein on gels was unaffected by treatment with neuraminidase, an enzyme that specifically removes terminal sialic acid residues from carbohydrate chains (data not shown). Our results indicate that elimination of glycosylation at either or both of the sites in the S1–S2 loop did not prevent the expression of active channels on the cell surface, indicating that glycosylation is not required for proper folding of the subunits, assembly of channels, or their transport to the plasma membrane.

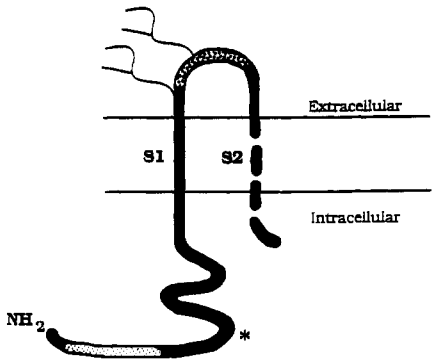

FIGURE 7: Extracellular location of the S1–S2 loop. A schematic drawing shows the sites of extracellular carbohydrate attachment at N259 and N263 in oocytes. The N-terminus is depicted as intracellular due to its role in inactivation (Hoshi et al., 1990; Zagotta et al., 1990). Therefore, the S1 segment spans the membrane. Hydrophobic segment S2 is shown dashed since our results do not address its location. Stippled regions indicate the approximate locations in the sequence of the peptide epitopes of the two antisera used to probe the immunoblots shown in Figure 2. The star (*) indicates the approximate location in the N-terminal region of a potential glycosylation site, N102, that is not modified in oocytes. The diagram is not drawn to scale.

DISCUSSION

The posttranslational modification of Shaker protein is interesting for several reasons. First, in membrane proteins, asparagine residues modified by glycosylation are generally thought to be located exclusively on the extracellular surface. Therefore, identification of the sites of posttranslational modification can be used to test models of membrane topology for channel subunits that have been derived by hydrophobicity analysis and electrophysiological assay. Glycosylation of the S1–S2 loop is strong evidence that this loop is extracellular, consistent with models for the topology of the Shaker subunit postulating a cytoplasmic location for the N-terminus and a transmembrane disposition for hydrophobic segment S1 (Figure 7). [There has been one report, however, of the presence of a small amount of N-linked carbohydrate on the cytoplasmic face of the α subunit of the Na,K-ATPase (Pedemonte et al., 1990).] The N-terminal region is known to be located on the cytoplasmic side of the membrane. This region plugs the mouth of the pore during inactivation, an effect that can be mimicked by an N-terminal peptide added to the intracellular face of the channel (Hoshi et al., 1990; Zagotta et al., 1990; Demo & Yellen, 1991). Therefore, glycosylation of the S1–S2 loop is biochemical evidence that the S1 segment, identified by hydrophobicity analysis, spans the membrane.

Second, glycosylation can serve as an indication of the transit of a membrane protein through the endomembrane system of cells. The initial stage of glycosylation occurs in the endoplasmic reticulum, generating a core carbohydrate structure that is trimmed and extended after the protein is transferred to the Golgi compartment (Hubbard & Ivatt, 1983; Rowling & Freedman, 1993). The immature, incompletely glycosylated protein detected in oocytes may correspond to a core glycosylated form of the protein from the endoplasmic reticulum. The predominant Shaker protein detected in oocytes is the completely glycosylated form, which is likely to represent the form expressed on the cell surface. We have not, however, determined what fraction of the protein in oocytes is on the cell surface.

Third, heterologous expression systems such as *Xenopus* oocytes are commonly used for analyzing the functional effects of site-directed mutations, yet the protein made in such systems has been little characterized. Finally, expression systems are a likely source of purified channel protein for detailed structural analysis, since some of them can provide a large amount of protein with a homogeneous subunit composition (Santacruz-Toloza et al., 1994). Thus, it is of interest to characterize protein that might be used for structural studies.

Glycosylation of Shaker Protein in Sf9 Cells. Sf9 cells infected with the Shaker recombinant baculovirus produce a triplet of immunoreactive proteins. The smallest band of the triplet, a 71 000-dalton protein, is unglycosylated, whereas the upper two bands contain N-linked oligosaccharides. By analogy with protein made in oocytes, the bands with relative molecular masses of 74 000 and 77 000 may represent Shaker protein N-glycosylated at one or two sites, respectively. Alternatively, the upper bands of the Shaker triplet may represent protein with different amounts of carbohydrate attached at only one site.

The glycosylation of the protein in this insect system may resemble that which occurs in flies. On immunoblots of protein from fruit flies, the Shaker protein is seen as a broad band with a relative molecular mass range of 69 000–80 000 daltons (D. M. Papazian, unpublished observations). This heterogeneity could be due in part to the existence of several different Shaker proteins with deduced molecular masses of 64 000–74 000 daltons that are encoded by alternatively spliced mRNAs (Schwarz et al., 1988, 1990). If the Shaker proteins made by flies contain N-linked oligosaccharides, not more than about 3000–6000 daltons of carbohydrate could be added. This would be similar to the amount added in Sf9 cells.

In Sf9 cells, much of the protein lacks N-linked oligosaccharides, since the unglycosylated band of the triplet is the most prominent one on immunoblots. Glycosylation of protein is normally a cotranslational event, so that full-length, unglycosylated forms of protein are not generally found. In Sf9 cells, however, a large amount of a heterologous protein is being expressed in cells infected with a lethal virus. Under these circumstances, the glycosylation apparatus is apparently unable to modify all of the Shaker protein. A similar result was obtained after expression of a functional Na–Ca exchanger using a recombinant baculovirus. Although this membrane protein is glycosylated in its native tissues, it is unglycosylated in Sf9 cells (Li et al., 1992).

Immunocytochemical labeling of Shaker-infected Sf9 cells indicates that a large pool of Shaker protein is intracellular (unpublished observations), which may include some or all of the unglycosylated protein. This intracellular pool of protein may be one reason why whole cell patch clamp analysis of Sf9 cells infected with the Shaker recombinant virus detects much less current than would be expected if all of the channel protein were expressed in active form on the cell surface (Klaiber et al., 1990). It remains to be determined whether the intracellular Shaker protein can be assembled into active channels. Since unglycosylated protein made by the N259Q + N263Q double mutant forms functional channels in oocytes, a lack of carbohydrate *per se* on the intracellular pool of protein in Sf9 cells may not prevent it from assembling into active channels. Rather, the large intracellular pool may accumulate because the machinery for transporting channels to the cell surface is saturated by the large amount of Shaker protein produced or is deficient due to the progress of the baculovirus infection.

Glycosylation of Shaker Protein in Oocytes. In contrast, to Sf9 cells, all of the Shaker protein made in oocytes appears to be glycosylated. Most of it exists as a heavily glycosylated band of about 113 000 daltons that is likely to represent the

mature, cell-surface form of the protein. Although the oligosaccharide is added to the same sites that appear to be modified in Sf9 cells, the structure, size, or composition of the carbohydrate added in oocytes is different, resulting in a large change in the migration of the protein on SDS–polyacrylamide gels. In oocytes, complete glycosylation increased the apparent molecular mass of the Shaker protein by about 40 000 daltons (Figure 3). This increase may not be due to the addition of 40 000 daltons of carbohydrate. Glycosylation is known to alter the hydrodynamic properties of glycoproteins so that their apparent molecular masses are increased by more than the mass of oligosaccharide actually added. The carbohydrate portion of glycoproteins may bind little or no SDS, resulting in anomalous migration on SDS gels. Alternatively, the large change in the mobility of the protein may be due to the attachment of highly-branched or charged oligosaccharide chains (Fish, 1975; Leach et al., 1980).

By analogy with other systems in which glycosylation has been studied, it is likely that the immature product contains core glycosylation attached in the endoplasmic reticulum and that the carbohydrate moieities of the mature product have been trimmed and extended in the Golgi compartment (Hubbard & Ivatt, 1983; Rowling & Freedman, 1993).

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