Both N- and C-terminal Regions Contribute to the Assembly and Functional Expression of Homo- and Heteromultimeric Voltage-gated K⁺ Channels

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The functional diversity of voltage-gated K⁺ channels may be partially determined by the mechanisms that permit or limit the assembly of molecularly diverse K⁺ channel subunits. To determine possible amino acid sequence domains required for subunit assembly and expression, we have constructed 15 N- and C-terminal interstitial or truncation deletion mutations in mKv1.1 (MBK1), a mouse Shaker-like K⁺ channel. We injected *Xenopus* oocytes with cRNA encoding each of these mutants and coinjected each mutant cRNA with cRNA for wild-type mKv1.3, another mouse Shaker-like K⁺ channel that can form heteromultimers with mKv1.1. We found that the last five amino acids of the C-terminus of mKv1.1 contribute to functional expression by (1) rescuing the function of mutants with a large truncation of the C-terminus ($\Delta 424$ –495), and (2) contributing to the slow inactivation kinetics (time constant of 2–3 sec) of wild-type mKv1.1 whole-cell K⁺ currents. All C-terminal deletion mutants were able to express at least as heteromultimers with mKv1.3, suggesting that the C-terminus is not required for channel assembly. In contrast, nine different interstitial or truncation mutants in which part of a highly conserved, large (80–99 amino acid residues) domain within the N-terminus had been deleted were unable to express either homomultimers or heteromultimers. The relatively small sizes and nonoverlapping distributions of the interstitial deletions enable us to suggest that the structural integrity of this entire N-terminal domain is required for subunit assembly and functional expression of this and probably other Shaker-like K⁺ channel proteins.

[Key words: potassium channel, subunit assembly, deletion mutant, oocyte, dendrotoxin, inactivation]

Several lines of evidence suggest that functional voltage-dependent K⁺ channels are composed of four subunits. Sequence analysis of clones from the Shaker locus (Tempel et al., 1987; Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988) revealed that the predicted protein was analogous to one of the four

internally homologous domains in functional voltage-dependent sodium and calcium channel α -subunits (Noda et al., 1984; Tanabe et al., 1987). Direct tests of subunit stoichiometry were provided by analysis of toxin block of one expressed Shaker clone (MacKinnon, 1991), and by construction of synthetic K⁺ channels linking various numbers of subunit domains (Liman et al., 1992). However, the molecular mechanisms by which K⁺ channel subunits assemble to form channel complexes are not understood. These undefined mechanisms allow the assembly of identical K⁺ channel subunits (homomultimers) into functional channels (Timpe et al., 1988), as well as the assembly of nonidentical subunits from the same gene subfamily (heteromultimers) (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). The combinatorial possibilities are apparently limited, however, since K⁺ channel subunits from different gene subfamilies appear not to assemble when coexpressed in *Xenopus* oocytes (Covarrubias et al., 1991; Li et al., 1992).

These observations raise an important question regarding the primary structural requirements for K⁺ channel subunit assembly and functional expression. All voltage-dependent K⁺ channels are thought to consist of cytoplasmic N- and C-terminal domains flanking a region consisting of six membrane-spanning domains and a pore region linked by small cytoplasmic or extracellular loops (Miller, 1991). At least one K⁺ channel type, rKv2.1 (drk1), can assemble and function in *Xenopus* oocytes with truncations that delete major portions of the N- and C-termini (VanDongen et al., 1990), implying that the transmembrane domains with minimal flanking residues are sufficient to enable subunit assembly and function. Using truncation deletions, Li et al. (1992) identified an amino acid sequence domain, which is highly conserved within the Shaker subfamily of genes, consisting of 114 residues in a hydrophilic region of the N-terminus of Shaker B (ShB) K⁺ channel subunit that is required to bind to the full-length ShB polypeptide.

We have taken a functional approach to address the question K⁺ channel subunit association and expression in *Xenopus* oocytes. We constructed a number of interstitial or truncation deletion mutants that together span major portions of the N- and C-termini of mKv1.1 (Tempel et al., 1988), a mouse member of the Shaker subfamily. We then tested the ability of these mutants to form functional channels when injected alone (presumably by forming homomultimers) or following coinjection with wild-type mKv1.3 (to allow formation of heteromultimers) (Chandy et al., 1990). We exploited the functional differences between these two Shaker-like channels to demonstrate that, indeed, wild-type mKv1.1 and mKv1.3 can form heteromul-

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timers with a probable subunit stoichiometry of four. We also found that interstitial deletion mutations that included a portion of a large domain within the N-terminus of mKv1.1 could not form and express functional channels either as homomultimers or as heteromultimers with mKv1.3. These results taken together with those of Li et al. (1992) suggest that this N-terminal domain is required for the assembly of Shaker-like subunits into functional K⁺ channels.

Materials and Methods

Generation of mutant cDNAs. Standard molecular techniques were used throughout. mKv1.1 (MBK1) and mKv1.3 (MK3) were cloned into an expression vector, pGEX, which contains plasmid sequences from pGem3Zf (Promega), 5' untranslated sequences from *Xenopus* β -globin, and a modified translational initiation region containing BglII, NcoI, and ApaI cloning sites (kind gift of D. Andrews, McGill University). For pGEX-1.1, the NcoI site was used to replace the β -globin initiation codon with that of mKv1.1. In pGEX-1.3, 36 base pairs (bp) of the 5' untranslated region endogenous to the K⁺ channel gene were subcloned into the BglII site of the pGEX vector. Mutants were generated in pGEX-1.1 using the following techniques.

(1) Linker mutagenesis: pGEX-1.1 was digested using two unique restriction endonucleases, a synthetic oligonucleotide linker being used to reclose the plasmid. The oligonucleotide linkers were designed to provide compatible overhanging ends while maintaining the open reading frame. Specific oligonucleotide sequences ranged in size from 13 to 64 bases and are available by contacting the Tempel lab. As an example, mutant Δ 53–159 was generated as follows: pGEX-1.1 was digested with BstBI and SacI, gel purified, and reclosed using a linker formed by annealing 23 base sense and 17 base antisense oligonucleotides. The linker contained AA48–52(FETQL) fused to AA160–163(PESS) and resulted in the deletion of AA53 to AA159. A similar strategy was used to construct mutants Δ 1-A-31, Δ 53–68, Δ 53–104, Δ 53–124, Δ 53–159, Δ 74–104, Δ 111–124, and Δ 111–159. All oligonucleotides were synthesized by the Molecular Pharmacology Core (U.W.) or by the Molecular Biology Core (VAMC), both of which use ABI DNA synthesizers.

(2) Cut-and-close mutagenesis: pGEX-1.1 was digested with two enzymes, the resulting ends being enzymatically modified with Klenow enzyme or mung bean Nuclease before reclosure. These techniques were used to construct mutants Δ 1-G-48 and Δ 127–166.

(3) Site-directed mutagenesis: pBS-1.1(9.4Sac), a subclone of pBS-1.1 that does not contain any N-terminal sequences, was mutagenized using polymerase chain reaction (PCR) (Ito et al., 1991) in order to introduce a novel SphI site beyond the conserved transmembrane region. pBS-1.1(9.4Sac/Sph) was used in turn to generate a frame-shift mutation (by filling and reclosing) that replaces the original 3' terminus of mKv1.1 with 10 nonsense amino acids followed by a stop codon (LSCSMLVLLT*) but does not truncate the transcript. The region from the BstXI site (in S4) to the end of this mutant subclone was used to replace the corresponding region in each of pGEX-1.1 and the previously constructed N-terminal deletion mutant Δ 131–159, thereby generating Δ 424–495 and the double mutant Δ 131–159 + Δ 424–495, respectively.

A combination of linker mutagenesis and PCR was used to generate Δ 427-H-490. For PCR the 5' primer included an SphI site and encoded LLTDV*. This primer in combination with T7 was able to amplify the 3' end of pGEX-1.1. The resulting amplification product was then subcloned into the SphI site of pBS-1.1(9.4Sac/Sph), followed by resubcloning into pGEX-1.1 at the BstXI site to generate Δ 427-H-490.

All mutated and PCR-amplified regions were sequenced on both strands using fluorescently labeled dideoxy terminators and Taq polymerase (Applied Biosystems) in a PCR-based sequencing protocol. The resulting sequences were analyzed by the Molecular Pharmacology Core using an ABI sequencer.

Oocyte expression. Plasmid DNAs were linearized with EcoRI for *in vitro* transcription of capped mRNAs (mCAP kit, Stratagene) using SP6 RNA polymerase. Mutant Δ 480–495 mRNA was obtained by digesting pGEXKv1.1 DNA with PvuII instead of EcoRI to linearize and truncate the template prior to transcription. Adult female *Xenopus laevis* were anesthetized by immersion in ice water for one-half hour, followed by surgical removal of two to four ovarian lobes. Pieces of ovary were washed in a calcium-free solution (OR2) containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 5 mM HEPES (pH 7.65). Single oocytes free from follicular cells and connective tissue were obtained by treatment

with 1 mg/ml collagenase (type A, Boehringer Mannheim) in OR2 at room temperature (22°C) for 3 hr. Healthy oocytes at maturation stages V and VI were selected and transferred to a modified Barth's solution (MBS) containing 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 2.4 mM NaHCO₃, 0.5 mM theophylline, 2.5 mM Na pyruvate, 0.1 mg/ml gentamicin, 0.01 mg/ml penicillin, 0.01 mg/ml streptomycin, and 10 mM HEPES (pH 7.4). Following 24 hr of incubation at 18°C, the oocytes were injected with 50 nl of 3–1000 pg/nl cRNA using a Drummond microinjector. Oocytes were maintained in MBS at 18°C and tested for current expression 2–6 d after injection.

Electrophysiology and data analysis. For whole-cell current measurements, a two-electrode voltage clamp (TEC01, NPI electronics) was used. Both the voltage recording and current electrode were filled with 3 M KCl and had resistances of 0.5–2.0 M Ω . The oocytes were placed in an 0.5 ml chamber and perfused continuously at 5 ml/min with frog Ringer's solution (FRS) consisting of 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES (pH 7.2). Transient capacitive and linear leakage currents were subtracted on line by the *P/4* procedure (Armstrong and Bezanilla, 1974) built into the data acquisition software (Instrutech ACQUIRE program). The currents were filtered at 1 kHz and sampled at 2 kHz. The oocyte membrane was clamped at –70 mV and membrane currents were elicited by 250 or 1000 msec command voltages from –70 to 50 mV in 10 mV increments. The time course of current inactivation during 1 sec voltage pulses was fit by the method of least squares with a single exponential function, and time constants of inactivation were derived from these functions using an exponential curve-fitting program. The adequacy of curve fits to single exponential functions was determined by eye, with complete superposition of the fitting function and the data required. For the fitting of drug-binding isotherms to concentration-inhibition data, the adequacy of the fit was statistically evaluated with the χ^2 goodness-of-fit test with a significance level set at $p = 0.05$. All values are expressed as mean $\pm$ SEM.

The “I” fraction of dendrotoxin (DTX-I) was purified from crude snake venom by a modification of the method of Benishin et al. (1988), and diluted in FRS to obtain the final concentrations of DTX-I with which the oocyte was perfused. To obtain concentration-inhibition data for DTX-I, a current-voltage (*I*–*V*) relation was obtained from a given oocyte in FRS, and 5 min following perfusion of DTX-I another *I*–*V* was sampled. This procedure was repeated for an ascending series of DTX-I concentrations. We have determined that, at the solution flow rate used, 5 min is an adequate time interval to obtain “steady state” current measurements in DTX-I. The peak potassium current at 50 mV was measured in the presence of each DTX-I concentration and normalized to the current obtained in control FRS at the same voltage.

To determine the expression ratios for mKv1.1 and mKv1.3 in the coexpression experiments, we injected the same concentrations of cRNA for mKv1.1 and mKv1.3, each alone, as were coinjected into cells from the same batch, and then determined the average peak current from at least 10 cells in each group. We could not simply coinject fixed ratios of concentrations of cRNA since, for a given concentration of cRNA, mKv1.1 expressed current to a greater degree than mKv1.3. We chose not to transcribe cDNA mixtures (Kavanaugh et al., 1992) because of the differences in translation initiation sequences for pGEX-1.1 versus pGEX-1.3. These differences may cause differential translational efficiency, thereby yielding inaccurate estimates of expression ratios based on cRNA or cDNA concentrations.

Results

mKv1.1 and mKv1.3 form heteromultimers

When cRNA encoding mKv1.1 was injected alone into *Xenopus* oocytes, K⁺ currents that activated between –40 and –50 mV and inactivated slowly were observed (Fig. 1A, Table 1). These currents were also sensitive to low concentrations of DTX-I (Figs. 1A, 2A). The concentration of half inhibition, K_i , for DTX-I was estimated to be 3 nM using the best fit of a standard drug-binding isotherm to the data ($N = 7$; Fig. 2A). In contrast, K⁺ currents observed following the injection of transcript for mKv1.3 activated between –40 and –30 mV and inactivated more rapidly than mKv1.1 (Fig. 1B, Table 1). Furthermore, mKv1.3 currents were virtually insensitive to DTX-I (Figs. 1B, 2A; Table 1). We never observed currents with these properties in uninjected oocytes ($N = 8$).

Figure 1. Membrane currents and DTX-I sensitivity for mKv1.1, mKv1.3, and both expressed together. *A*, K^+ currents recorded from an oocyte injected with mKv1.1 cRNA in control saline, and following bath application of 10 nM DTX-I. *B*, The same for mKv1.3; the trace in the presence of 10 nM DTX-I is completely superimposed on the control trace. *C*, Currents following the coinjection of mKv1.1 and mKv1.3 in a 1:2 ratio, respectively (see Materials and Methods for ratio determination). The holding and command potentials were -70 and 50 mV, respectively. The concentrations of cRNA injected were (*A*) 0.25 ng/oocyte, (*B*) 0.67 ng/oocyte, and (*C*) 0.08 ng mKv1.1, 0.67 ng mKv1.3/oocyte. The time calibration in *A* applies to all traces.

We have taken advantage of differences in DTX-I sensitivity and inactivation kinetics to provide evidence for heteromultimer formation between mKv1.1 and mKv1.3. We observed that when mKv1.1 and mKv1.3 were coexpressed in a 1:2 ratio, respectively, K^+ currents appeared with DTX-I sensitivity approaching that of mKv1.1 alone (Figs. 1*C*, 2*A*). The estimated

Figure 2. Deviation of DTX-I sensitivity of K^+ currents in coinjected oocytes from an additive model. *A*, DTX-I concentration-inhibition data for mKv1.1 (squares), mKv1.3 (triangles), and when both were coexpressed in a 1:2 ratio (circles). The dashed curve represents output from an additive model that assumes that mKv1.1 and mKv1.3 express only as homomultimers (model equation given in Results). The data were taken from a total of seven oocytes for mKv1.1, 11 for mKv1.3, and four for both coinjected. The solid curves represent the best-fitting drug-binding isotherm to the data points in each case. *B*, The same concentration-inhibition data for the coinjected cells shown in *A* (circles) are compared to the results for the additive model with two possible expression ratios: 1:1 and 4:1 mKv1.1 to mKv1.3 (labeled dashed curves).

K_i for the coinjection data shown in Figure 2 was 14 nM ($N = 4$). If mKv1.1 and mKv1.3 are expressed only as homomultimers, then an additive model consisting of DTX-I-binding isotherms for both mKv1.1 and mKv1.3 should adequately de-

Table 1. Properties of mKv1.1, mKv1.3, heteromultimers, and two C-terminal truncation mutants of mKv1.1 expressed in *Xenopus* oocytes

	K_i (DTX-I) (nM)	Inactivation tau (msec)	Prepulse inactivation		
			k (mV/e-fold)	$V_{1/2}$ (mV)	F_{noninact}
mKv1.1	3 (7)	2337 ± 176 (7)	4.9 ± 0.3	-34 ± 1.6	0.71 ± 0.04 (9)
mKv1.3	4533 (11)	694 ± 61 (18)	3.5 ± 0.1	-26 ± 1.0	0.26 ± 0.02 (15)
1 mKv1.1 to 2 mKv1.3	14 (4)	1843 ± 264 (4)	4.2 ± 0.4	-24 ± 1.9	0.54 ± 0.03 (5)
mKv1.1Δ480–495		3810 ± 166 (4)			
mKv1.1Δ427–H-490		2386 ± 216 (5)			

K_i values for DTX-I were determined from the best-fitting binding isotherms to averaged concentration-inhibition data. All inactivation time constants were obtained at 20 mV. Prepulse inactivation was assessed by applying a 5 sec prepulse to voltages between -90 and 10 mV in 10 mV increments followed by a 250 msec test pulse to 20 mV from a holding potential of -70 mV. The prepulse inactivation parameters, k (slope factor) and $V_{1/2}$ (voltage at which half of the completely inactivating fraction of the total current inactivates), were determined from the best-fitting Boltzmann functions, and F_{noninact} refers to the fraction of the total K^+ current that did not inactivate using this protocol. The total number of oocytes for each parameter, or parameters in the case of prepulse inactivation, is given in parentheses.

Figure 3. DTX-I data predict a channel subunit stoichiometry of 4. The analytical approach of MacKinnon (1991) was applied to the DTX-I data shown in Figure 2. The ordinate variable is based on the left side of Equation 5 from MacKinnon (1991), with $f_{1.3} = 0.67$ (2 mKv1.3 to 1 mKv1.1 expression ratio), and U_{mix} and $U_{1.3}$ corresponding to the unblocked fraction of current for the coinjected cells ($N = 4$) and cells injected with transcript for mKv1.3 alone ($N = 11$), respectively. The curve is independent of the data and derived from the right half of Equation 5 assuming a subunit stoichiometry of 4, which provided a better fit to the data than the three or five subunit cases (not shown). Both the data and theoretical curve converge on a value of 4. Higher concentrations of DTX-I were not used since they usually killed the oocyte.

scribe the data from the coinjected oocytes. We used the following equation to implement the additive model:

$$U = F_{1.1}(1 - (X^n/X^n + K_{1.1}^n)) + (1 - F_{1.1})(1 - (X^n/X^n + K_{1.3}^n)),$$

where U is the unblocked fraction of K⁺ current, $F_{1.1}$ is the fractional expression of mKv1.1 ($F_{1.1} = 0.33$ for the data shown in Fig. 2), X is the DTX-I concentration, n is the Hill coefficient, and $K_{1.1}$ and $K_{1.3}$ are the concentrations of half-inhibition for mKv1.1 and mKv1.3 by DTX-I, respectively (Table 1). The values for the Hill coefficients used, derived from concentration-inhibition data for oocytes injected with mKv1.1 or mKv1.3 alone, were 0.63 and 0.72, respectively. The model outputs, assuming a 1:2 expression ratio for mKv1.1 and mKv1.3, for four concentrations of DTX-I are given in Figure 2A. It is clear that the data deviated significantly from the additive model, in that they displayed much greater DTX-I sensitivity than the model would predict. This approach assumes that we have accurately determined the expression ratio for mKv1.1 to mKv1.3. We used an analytical approach devised by MacKinnon (1991) and our DTX-I data to obtain an estimate for the K⁺ channel subunit stoichiometry (see below). Since the results obtained with that approach are very sensitive to the accuracy of the expression ratio estimate for the two types of subunits, it suggests that our method for determining this was appropriate. To determine if any output from this additive model could yield results similar to the observed data, we altered the expression

ratio (the weighting of each binding isotherm, $F_{1.1}$ in the equation above) to yield the model result best fitting the data (Fig. 2B). The best-fitting additive model corresponds to an expression ratio of 4 mKv1.1 to 1 mKv1.3. Although this is a statistically adequate fit ($p > 0.05$), it overestimates the DTX-I block at the two lowest concentrations. More importantly, this expression ratio greatly deviates from the measured ratio. The peak currents for mKv1.1 and mKv1.3 cRNA, when each are injected alone in oocytes from the same batch, were $1.41 \pm 0.17 \mu\text{A}$ ($N = 8$) for mKv1.1 and $2.89 \pm 0.28 \mu\text{A}$ ($N = 13$) for mKv1.3. Hence for the additive model to hold, mKv1.1 would have had to be expressed to a much higher degree (4:1) in the coinjected oocytes, relative to mKv1.3, than was observed for the separately injected oocytes (1:2). Similar results were obtained in nine other coinjected oocytes from two separate batches.

Since the properly weighted additive model did not adequately describe the data, the most likely alternative hypothesis is that heteromultimeric K⁺ channels, consisting of both mKv1.1 and mKv1.3 subunits, can form and have DTX-I sensitivity intermediate between mKv1.1 and mKv1.3. Furthermore, the results suggest that the DTX-I sensitivity of most if not all types of heteromultimers formed are much closer to that of mKv1.1 than to mKv1.3.

Differences in the kinetics of inactivation between mKv1.1 and mKv1.3 provided an independent variable for analyzing the formation of heteromultimers. For the four coinjected oocytes previously mentioned, the observed time constant of inactivation was 1843 ± 264 msec (measured at 20 mV), intermediate between mKv1.1 and mKv1.3, but much closer to that of mKv1.1 (Table 1). The sum of two exponential terms, each describing the inactivation kinetics of mKv1.1 and mKv1.3 and weighted by their respective expression ratios, was fitted to the currents elicited by 1 sec command pulses to 20 mV for each of these coinjected oocytes. In each case, the inactivation time course predicted by the additive model was considerably faster than the observed data, and therefore could not provide an adequate fit to the data (data not shown). As with the DTX-I data, the additive model could provide adequate fits to the observed inactivation time courses only if the expression ratios for mKv1.1 and mKv1.3 in the coinjected oocytes were assumed to be greatly weighted toward much higher expression of mKv1.1 than that observed in separately injected oocytes.

Subunit stoichiometry

Using a novel analytical approach, and a point mutation in ShB K⁺ channel subunits that renders them virtually insensitive to charybdotoxin, MacKinnon (1991) was able to estimate the subunit stoichiometry for this K⁺ channel in *Xenopus* oocytes. We applied the assumptions and equations of that approach to the DTX-I data shown in Figure 2A, since they are analogous to the charybdotoxin data. The results are shown in Figure 3. The averaged data from the four oocytes appear to converge on a value of 4 as DTX-I concentration is increased. The theoretical curve, which assumes a subunit stoichiometry of 4, provided a very good fit to the data, while the curves for the three or five subunit cases did not (data not shown). The data provide further support for the hypothesis that voltage-gated K⁺ channels are tetramers.

Expression of N- and C-terminal deletion mutants of mKv1.1

A total of 11 N-terminal, three C-terminal, and one combined N- and C-terminal deletion mutants of mKv1.1 were tested in

Figure 4. Deletion mutants and summary of results. A schematic of the coding region of mKv1.1 is shown at the top, with putative membrane-spanning domains (S1–S6) and pore region (H5) shaded. The N- and C-terminal domains, and extracellular and cytoplasmic loops joining membrane-spanning regions are not shaded. Schematic diagrams of 15 deletion mutants tested in the study, with the corresponding amino acids deleted, are shown below. Amino acids are numbered from the beginning of the coding region. The single letters inserted for three of the mutants correspond to amino acids that were inserted to restore the restriction sites and keep the nucleotide sequence of the original cDNA construct in frame (see Materials and Methods). The G corresponds to glycine, A to alanine, and H to histidine. A summary of the results is shown on the right, with “homomult” denoting expression as a homomultimer, that is, injected alone, and “heteromult” corresponding to expression as a heteromultimer when coinjected with wild-type mKv1.3.

this study. Figure 4 depicts all of these mutants with their relative positions and sizes in schematic form, as well as the amino acid residues that were deleted from the N-terminus, C-terminus, or both. The cRNA transcribed from the mutant cDNAs was always injected undiluted to optimize detection of expression. The amount of cRNA injected, estimated from optical density readings, was 25–50 ng/oocyte. In every case, transcript size and quality were confirmed by formaldehyde denaturing gel analysis.

To determine possible structural requirements for assembly and expression of mKv1.1 as a homomultimer or heteromultimer with mKv1.3, we injected cRNA encoding the mutants alone or with a fixed concentration of cRNA encoding wild-type mKv1.3 (0.17 ng/oocyte). The same concentration of mKv1.3 cRNA alone was injected in oocytes from each batch as a control to assess expression of mKv1.3. The average K^+ current in oocytes injected with 0.17 ng of mKv1.3 cRNA was $1.50 \pm 0.24 \mu A$ measured at 50 mV ($N = 21$). To determine whether mKv1.3 expression was constant over the several months of the study, we split these data into two roughly equal groups corresponding to the first and second half of the study. The average K^+ current for the first half was $1.56 \pm 0.30 \mu A$ ($N = 10$) and for the second half was $1.45 \pm 0.39 \mu A$ ($N = 11$). Therefore, neither the stability of the transcript nor the ability of the oocytes to express K^+ current following injection of a fixed concentration of cRNA encoding mKv1.3 varied significantly over the course of the study.

The expression results obtained when cRNA encoding each deletion mutant was injected alone to test for homomultimer formation are summarized in Figure 4. Each data point is based on recordings from 4–12 oocytes for each mutation. Of the N-terminal mutants, only the Δ1-A-31 mutant, in which the first 31 amino acid residues were deleted, yielded measurable K^+ currents. All others displayed currents that were indistinguishable from those observed in uninjected oocytes (data not

shown). For the C-terminal mutants, the Δ480–495 and Δ427-H-490 each yielded K^+ currents, while the Δ424–495 mutant did not. The N- and C-terminal double mutant, Δ131–159 + Δ424–495, also did not express K^+ current, as would be predicted from the results of each separate mutation.

The results of the experiments in which cRNA encoding deletion mutants was coinjected with wild-type mKv1.3 to test for heteromultimer formation are shown in Figure 5 for seven of the 11 N-terminal and all of the C-terminal and double mutants. The averaged data are based on measurements from 3–12 oocytes for each mutation. Figure 5A depicts the percentage block of a concentration of DTX-I (10 nM) that distinguishes between mKv1.1 and mKv1.3 (upper two bars; see also Fig. 1A,B). The third bar corresponds to the DTX-I sensitivity of K^+ currents from oocytes coinjected in a 1:2 ratio of mKv1.1 and mKv1.3, respectively. The N-terminal mutants are grouped in a linear sequence down from the start of the coding region to within two residues of the first putative membrane-spanning domain. For this group, the Δ1-A-31 (first) and Δ131–159 (last) mutants were able to express as heteromultimers, as determined from the intermediate DTX-I sensitivity of the resulting K^+ currents (Fig. 5A). The remaining five mutants, Δ1-G-48 to Δ127–166 (central), displayed currents with DTX-I sensitivity similar to mKv1.3 alone. As these mutants neither added mKv1.1-like increased DTX-I sensitivity nor significantly reduced expression of mKv1.3, as determined by comparing K^+ current amplitude in the coinjected cells with cells injected with mKv1.3 cRNA alone (data not shown), we conclude that they were unable to assemble and express as functional heteromultimers. The remaining four N-terminal mutants (Fig. 4), which are not shown in Figure 5A, each encompass larger deletions within the domain spanned by the five nonexpressing mutants, and also did not express as heteromultimers. All three C-terminal mutants, which together span from 13 amino acid residues from the sixth putative membrane-spanning domain to the

Figure 5. DTX-I and inactivation time constant data summary for the mKv1.1 deletion mutants coexpressed with mKv1.3. *A*, The average block (%) of K⁺ current by 10 nM DTX-I is shown for wild-type mKv1.1, mKv1.3, and when transcripts encoding both are coinjected in a 1:2 ratio, respectively. The dashed line corresponds to the predicted block for an additive model assuming a 1:2 expression ratio for mKv1.1 and mKv1.3, respectively. All of the data following these three groups are from oocytes coinjected with cRNA encoding deletion mutants and wild-type mKv1.3. The N-terminal deletion mutants are grouped together and sequentially span from the start site to within two amino acid residues of the first putative membrane-spanning domain. The three C-terminal mutants are grouped below, with a combined N- and C-terminal mutant shown last. The concentrations of deletion mutant cRNA injected were variable, but were always undiluted (25–50 ng/oocyte), while the concentration of mKv1.3 cRNA was always 0.17 ng/oocyte. *B*, The average inactivation time constants, in msec (see Materials and Methods), sampled in FRS, are shown for the same data set depicted in *A*.

end of the coding region, were able to express as heteromultimers (Fig. 5*A*). The double mutant Δ131–159 + Δ424–495 was also able to associate with mKv1.3, as did each mutation separately.

This pattern of results was perfectly corroborated by the inactivation time constant data (Fig. 5*B*). Again, inactivation time constants for wild-type mKv1.1, mKv1.3, and following coinjection in a 1:2 ratio, respectively, are given by the first three bars. The Δ1–A–31 and Δ131–159 N-terminal mutants displayed intermediate inactivation time constants characteristic of heteromultimers when coinjected with mKv1.3, while the other five mutants did not. For the C-terminal mutants, the inactivation time constants for Δ424–495 and Δ427–H–490 coinjected with mKv1.3 were similar to those displayed by heteromultimers. Similarly, the double mutant Δ131–159 + Δ424–495, when coexpressed with mKv1.3, yielded K⁺ currents that inactivated with an average time constant characteristic of heteromultimers of each separate mutation (Fig. 5*B*). The high correlation between the DTX-I data and the inactivation time constant data make it unlikely that the pattern of results observed with DTX-I was due to altered DTX-I sensitivity in the mKv1.1 mutants. However, the Δ480–495 mutant coinjected with mKv1.3 yielded unusually large inactivation time constants (3128 ± 288 msec at 20 mV, $N = 5$), larger even than wild-type mKv1.1. Furthermore, the average inactivation time constant of this mutant, expressed as a homomultimer, was 3810 ± 166 msec ($N = 4$). This is almost twice as large as the inactivation time constant for mKv1.1 (Table 1), and therefore represents an altered phenotype resulting from this mutation. That the inactivation time constant for this mutant coinjected with mKv1.3 was somewhat smaller than the mutant expressed alone suggests that it was able to form heteromultimeric channels with mKv1.3. A summary of the expression results for all of the deletion mutants coinjected with mKv1.3 is given in Figure 4.

Discussion

As a first step to evaluating the possible role of the N- and C-terminal domains in the ability of mKv1.1 to express functional K⁺ channels, we constructed a series of interstitial and truncation deletion mutants that together spanned virtually all of each domain. After establishing that wild-type mKv1.1 and mKv1.3 were able to form heteromultimers with a probable subunit stoichiometry of 4, we injected cRNA encoding each mutant alone or in combination with wild-type mKv1.3 to determine the ability to form functional homomultimers or heteromultimers in *Xenopus* oocytes. We found that all of the C-terminal mutants tested could express functional channels as either a homomultimer or heteromultimer or both. In contrast, only two of the 11 N-terminal mutants could do so.

In contrast to the dominance of fast, N-terminal-mediated inactivation observed when rapidly inactivating Shaker A and slowly inactivating mutant Shaker B subunits are coexpressed (Isacoff et al., 1990), we observed that mKv1.1 provides the dominant phenotype for slow inactivation kinetics. This was also the case for DTX-I sensitivity, since both toxin sensitivities and the inactivation time constants of currents from coinjected oocytes were much closer to mKv1.1 than to mKv1.3. In this regard, it is interesting that differences in both DTX-I sensitivity and inactivation kinetics between the rat homologs of mKv1.1 and mKv1.3 have been attributed, at least in part, to the identity of one amino acid residue between S5 and S6, near the putative mouth of the channel pore. A histidine residue at position 401

Name	C-term. sequence	Size	Species	Reference
ShA	NNAMAVSIET TDV *	616	<i>Drosophila</i>	Tempel et al., 1987
ShB	NNALAVSIET TDV *	656	<i>Drosophila</i>	in Drewe et al., 1992
AK01	NPGSDYGLE TDV *	515	<i>Aplysia</i>	in Drewe et al., 1992
XSh2	NYVNITKML TDV *	499	<i>Xenopus</i>	in Drewe et al., 1992
1.1	NCVNKSKLL TDV *	495	Mouse	Tempel et al., 1988
1.2	NYVNITKML TDV *	499	Mouse	Chandy et al., 1990
1.3	SCVNIKKIF TDV *	530	Mouse	Chandy et al., 1990
1.4	NCSNAKAVET TDV *	655	Rat	Stühmer et al., 1989
1.5	ALCLDTSRET DL *	602	Rat	Swanson et al., 1990
1.6	RAYAEKRM LETV *	529	Mouse	Migeon et al., 1992

Figure 6. Distal C-terminal amino acid sequence lineup for several Shaker subfamily members. The amino acid sequence of the distal C-termini of several members of the Shaker subfamily from a variety of species are given (standard single letter code), as well as the predicted size of the polypeptide (total amino acid residues). Note the strong sequence conservation of the last three residues (identical residues in bold type).

results in less sensitivity to DTX-I and faster inactivation than when tyrosine resides at this position (Busch et al., 1991; Hurst et al., 1991).

A possible molecular interpretation of dominant phenotypes is suggested by considering the subunit composition of the resulting channels in a mixing experiment such as this. Let us assume that subunits assemble randomly, and therefore binomial statistics accurately describe the assembly of mKv1.1 and mKv1.3 subunits into channels composed of four subunits with all possible subunit compositions (1 mKv1.1 to 3 mKv1.3, 2 mKv1.1 to 2 mKv1.3, etc). With the 1 mKv1.1 to 2 mKv1.3 expression ratio displayed in this experiment, we would predict that 80% of the channels have at least one mKv1.1 subunit, but that only about 40% have two or more mKv1.1 subunits. Therefore, in order for a particular mKv1.1 property to be dominant as measured by whole-cell recording, it must be the case that a channel need have only one mKv1.1 subunit to express the mKv1.1 phenotype. Analogous to studies using charybdotoxin (MacKinnon, 1991), it may be that one DTX-I-sensitive subunit provides a sufficient binding site to confer nearly full DTX-I sensitivity on a channel complex. In the case of slow inactivation kinetics, it is possible that a channel's inactivation properties are largely determined by the kinetics contributed by its "slowest" subunit.

Synthesis and stability of mutant subunits

When cRNA encoding each of the deletion mutants was coinjected with transcript encoding mKv1.3 (heteromultimer expression), the pattern of results was not identical to what was observed with each deletion mutant injected alone (homomultimer expression, Fig. 4). That is, one N-terminal (Δ 131–159) and one C-terminal (Δ 424–495) deletion mutant could not express as homomultimers, but were able to express as heteromultimers with mKv1.3 (Fig. 5). These data suggest that protein is made from these deletion mutants and is available to assemble with intact mKv1.3 subunits. In contrast, several N-terminal deletions showed no effect on the expression of mKv1.3 upon coinjection. The results of Li et al. (1992) suggest that mutants lacking these N-terminal regions would not bind biochemically to intact channel subunits. Thus, our inability to detect mKv1.1-like currents from these deletion mutants is likely to result from their failure to assemble either with mKv1.3 (heteromultimers) or with each other (mutant homomultimers). Further support for this conclusion comes from our observation that these mutants did not block or reduce expression of mKv1.3 in a dominant negative fashion, as would be expected had the N-terminal region alone been expressed (Isacoff et al., 1990).

Comparing all of our deletion constructs, our results indicate

that the location of the deletion was more important than its size. For example, both the Δ 53–124 and Δ 424–495 mutants each deleted 71 amino acid residues in the N- and C-termini, respectively. However, the C-terminal mutant was able to express as heteromultimers, whereas the N-terminal mutant did not display any form of expression. The Δ 427–H-490 mutant consisted of a 63 amino acid deletion in the C-terminus that was able to express as both homomultimers and heteromultimers. This deletion was larger than seven of the nine N-terminal deletions that did not display any form of expression. It was therefore not the case that the probability of expression was simply related to the size of the deletion.

Functional role of the C-terminus

The importance of the distal C-terminus in Shaker-like K⁺ channel proteins for their stability and function is suggested by the remarkable amino acid sequence conservation displayed within this region between channel proteins from phylogenetically diverse species, such as flies, mollusks, amphibians, and mammals (Fig. 6; Drewe et al., 1992). With this conservation in mind, it is interesting to note that our data suggest the distal C-terminus (i.e., the last five amino acid residues) may contribute to K⁺ channel stability and function. The Δ 424–495 C-terminal mutant channels were able to express only as heteromultimers, whereas the Δ 427–H-490 mutant could express as both homomultimers and heteromultimers. Hoshi et al. (1991) had suggested the possible importance of the last five amino acid residues of the C-terminus in the expression of Shaker A and B K⁺ channels. These residues are clearly not required for channel function, since the Δ 480–495 mutant was able to express as homomultimers and heteromultimers. However, the presence of residues 490–495 does restore homomultimeric function to the large C-terminal mutant Δ 424–495. It is therefore possible that these residues enhance the protein stability of what would otherwise be a severely truncated protein, but that this could also be accomplished by associating with wild-type subunits (mKv1.3).

Our results also suggest that the distal C-terminus may play a role in the slow inactivation exhibited by mKv1.1. The Δ 480–495 mutant, expressed as a homomultimer, exhibited considerably slower inactivation than wild-type mKv1.1, whereas the time constants for the Δ 427–H-490 mutants, also expressed as homomultimers, were nearly identical to mKv1.1 (Table 1). This form of inactivation (time constant of 2–3 sec) is slower than other types of slow inactivation that have been identified and attributed to the presence of specific amino acid residues in other regions of Shaker or Shaker-like K⁺ channel proteins (Busch et al., 1991; Hoshi et al., 1991). We have not yet con-

Figure 7. N-terminal domain required for expression of mKv1.1. The amino acid sequences of the N-terminus for mKv1.1 and mKv1.3 are given (standard single-letter code), with identical residues denoted with a dash. The domain suggested by our results to be required for expression of mKv1.1 is shown as a bar over the mKv1.1 sequence. The uncertainty in the size of this domain is denoted by crosshatchings. For comparison, the domain determined by Li et al. (1992) is also shown as a dashed line over the mKv1.1 sequence.

structed and tested a $\Delta 491$ –495 mutant mKv1.1, and therefore do not know that this form of slow inactivation is specifically mediated by the distal five C-terminal residues. Nevertheless, it is intriguing that several kinetically distinct mechanisms of K⁺ channel inactivation may be mediated by distinct regions of the channel protein, and involve conformation-dependent interactions between predicted transmembrane and hydrophilic regions (Timpe et al., 1988; Iverson and Rudy, 1990; Busch et al., 1991; Hoshi et al., 1991; Stocker et al., 1991).

Functional expression requires a specific N-terminal domain

The pattern of results obtained for the N-terminal deletions (Fig. 5) suggests a more specific conclusion regarding a functional role for the highly conserved central domain of the N-terminus of these Shaker-like subunit proteins (Fig. 7). The $\Delta 1$ -A-31 mutant, in which the first 31 amino acids were deleted, was able to express as both homomultimers and heteromultimers. The $\Delta 131$ –159 mutant expressed only as a heteromultimer. The five relatively small interstitial deletion mutants that span from residue 48 to 127 between these two mutants did not display any form of expression, nor did any of the four mutants that encompass larger deletions within this region (Fig. 4). These results alone do not enable us to conclude that mutants that did not express were defective in the ability to assemble. However, our data and the results of Li et al. (1992) taken together suggest that a central domain within the N-terminus, which is as small as 80 residues and as large as 99 residues, is required for assembly and functional expression of mKv1.1 in any form (homomultimers or heteromultimers). The uncertainty in the size of this domain is related to the resolution offered by the relative sizes of the deletions used. We have delineated this domain within the N-terminus, with its size uncertainty, in Figure 7. The amino acid sequence of the N-terminus of mKv1.3 is aligned with that of mKv1.1 to illustrate the large degree of amino acid sequence identity between the two channel subunit proteins in this region. This N-terminal domain is similar to that which was identified by Li et al. (1992) for Shaker gene subfamily K⁺ channel subunit assembly. Our results suggest, however, that this domain is somewhat shorter than the one proposed by them, as our linker-mutagenesis approach enabled us to design specific interstitial deletion constructs that could more closely define the critical region. For example, $\Delta 1$ -A-31 was designed specifically to leave intact the beginning of the N-terminal region that is highly conserved between mKv1.1 and mKv1.3 (Fig. 7). This mutant, but not the next larger one ($\Delta 1$ -G-48) which deleted 17 additional

residues in this region, was able to express functional channels. The relatively small size and nonoverlapping distribution of our nine interstitial deletions provide the novel and important insight that the structural integrity of this entire region is important for functional expression of mKv1.1. Another functional test for the role of the N-terminus in subunit assembly was provided by Li et al. (1992), who found that heteromultimers across two K⁺ channel subfamilies can form when the N-terminal region in Shaker B is incorporated at the N-terminus of a chimeric Shaker B-drk 1 (rKv2.1) channel.

In a study similar to ours, VanDongen et al. (1990) found that deleting the first 139 amino acid residues of the 182 residue N-terminus of rKv2.1 (drk1) resulted in functional, albeit modified, K⁺ channels in *Xenopus* oocytes. We found that nine much smaller deletions that included homologous regions of the N-terminus of mKv1.1 resulted in no functional expression. These differences imply that our results are specific to members of the Shaker subfamily of K⁺ channel genes, and conversely that the results of VanDongen and colleagues may be specific to the Shab subfamily. In this regard, it is interesting to note that the largest C-terminal deletion reported by VanDongen and colleagues left intact 125 residues flanking S6, the last putative membrane-spanning domain. The initial 90 residues in this region are well conserved (96%) among various Shab-like genes (Pak et al., 1991; Hwang et al., 1992). It is therefore possible that a portion of the C-terminus of K⁺ channels encoded by Shab-like genes may be critical for subunit assembly, analogous to the proposed N-terminal domain required for this function in K⁺ channel subunits encoded by Shaker-like genes. The role of transmembrane domains in permitting or directing the assembly of voltage-gated K⁺ channels is unknown. It will be interesting to determine the relative weight of the contributions of the transmembrane domains compared to those of cytoplasmically located domains in the mechanisms that permit and limit assembly of specific K⁺ channel subunits.

Among other membrane proteins, aqueous as opposed to transmembrane domains appear to be critical for mediating subunit assembly. For example, the N-terminus, but not transmembrane domains, is required for assembly of some muscle ACh receptor subunits (Yu and Hall, 1991; Verrall and Hall, 1992). The β -subunit of the Na,K-ATPase, is thought to possess an N-terminal cytoplasmic domain and a C-terminal ectodomain. The specificity of assembly of the β -subunit with the α -subunit residues in the C-terminal ectodomain of the β -subunit, but not its transmembrane domain (Renaud et al., 1991).

There exist other examples of oligomeric membrane proteins in which specific interactions between aqueous domains are required for subunit assembly and stability (Hurtley and Helenius, 1989). Our results suggest that for K⁺ channel subunits encoded by genes from the Shaker subfamily, aqueous domains, especially the N-terminus, play a critical role in channel assembly.

Note added in proof: Since the revision of this manuscript, Shen et al. (Neuron 11:67–76, 1993) have reported data corroborating our finding that the N-terminus plays an important role in the assembly of Shaker-like K channels.

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