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Characterization of a Regulatory Region in the N-Terminus of Rabbit Kv1.3

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The N-terminus of rabbit Kv1.3 contains a signal sequence which regulates expression of Kv1.3 proteins in the plasma membrane. Removal of an N-terminal region (aa3-39) produced an increase in expressed K^+ current. Progressive deletion at the N-terminus demonstrated that the shortest deletion required for the elevation of K current is D6-34. Since the functional signal sequence must include both ends of the peptide segment aa6-34 where charged residues are densely distributed, it is conceivable that this N-terminal signal sequence is related to charge or its associated hydrophilicity. Removal of two charged residues (31R, 33E) through amino acid substitution which converts deletion construct D3-27 to D3-27* effectively raises the amplitude of expressed current, further indicating the importance of charged residues. With the use of a mutated dynamin and a soluble N-terminal peptide, we also revealed that the N-terminal signal sequence may not act through the endocytotic pathway. © 1998 Academic Press

Shaker-related K^+ channels are composed of four identical α-subunits, each of which contains a cytoplasmic amino terminus, six transmembrane segments, a pore region, and a cytoplasmic carboxyl terminus. These channels arise from a common ancestor and can be divided into four well-defined subfamilies, *Shaker, Shab, Shaw* and *Shal*. One member of *Shaker* subfamily K^+ channels, Kv1.3, plays an important role in development of human lymphocyte functions (1) and in maintenance of renal K^+ homeostasis (2). Kv1.3 is the most abundant channel in T lymphocytes. Two lines of evidence strongly suggest that elevation of Kv1.3 current is required for activation and proliferation of T lymphocytes. Firstly, magnitude of Kv1.3 current is increased by up to 10 to 15-fold following mitogen-induced activation of human and murine T lymphocytes (1, 3, 4). Secondly, specific blockers of Kv1.3 channel inhibit cellular events associated with T lymphocyte activation, including secretion of interleukin-2 and mitogen-induced proliferation of T-lymphocytes. However, the molecular mechanism that mediates the increase in Kv1.3 current during T lymphocyte activation process is still not understood.

The N-terminus of Kv1.3 channel contains a conserved T1 recognition site which may mediate the assembly of K^+ channel subunits into functional tetramers (5–8). In addition, we have demonstrated that a region immediately preceding the T1 recognition site contains a signal sequence that regulates the expression of K^+ channel proteins in the plasma membrane at least in rabbit Kv1.3 (5).

In the present study, we further examined the functional role of this N terminal region preceding the T1 recognition site. Site-directed mutagenesis is used to search for critical N-terminal amino acids associated with the expression of K^+ channel. We find that the effect of the N-terminus may be determined by charge or its associated hydrophilicity in the N terminus. The mechanism of increased channel expression is further explored by blocking endocytotic pathway with the use of a mutated dynamin and a soluble N-terminal peptide.

MATERIALS AND METHODS

Generation of mutant channels. A similar strategy was used to generate a series of deletion mutations D3-20, D3-27, D3-34, D3-36, D30-39, D3-42, and D3-52. Briefly, the rabbit Kv1.3 channel gene was cloned into pBluescript (Stratagene, San Diego, CA) as previously described (2). The polymerase chain reaction (PCR) was employed to create deletion mutations. The sense primer contained a T7 polymerase site, coding sequences for amino acid 1-2, and 20 bases flanking 3′ side of deletion. The antisense primer contained a polyA tail and additional 20 bases matching a sequence in 3′ untranslated region flanking the stop codon. PCR of 50 μL contained 1 μL template, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, dATP, dCTP, dGTP, dTTP 0.2 mM each, 1.0 μM primers and 2.5 Unit Taq DNA polymerase (Gibco-BRL). Fifty cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) were performed with a Robocycler (Stratagene). Control reactions without template were included for each PCR amplification experiment. RT-PCR products were purified from agarose gel using Elu-Quik gel-purification kit (Schleicher & Schuell). The

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products were end-filled using Klenow, and then cloned into a pBluescript vector (Stratagene). The identity and orientation of all constructs were verified by Sanger sequencing (9). D3-27* was constructed based on D3-27. A second round of PCR was used to introduce additional replacement of amino acids, thus converting D3-27 to D3-27*.

The deletion mutation D27-34, D20-34, D12-24, D6-34 were generated by double PCR techniques reported by Shimada (10). Briefly, the first round of PCR was used to amplify a short fragment containing the desired mutation, then the second round of PCR was performed to amplify the whole gene containing the desired mutation. The constructs were then cloned into pBluescript vector and sequenced by Sanger sequencing.

Constructs for dynamin and N-terminal peptide aa1-39. Constructs for wild-type dynamin and a mutated dynamin bearing point mutation at amino acid 44 (K44A) were gifts from another laboratory (11). The construct for the soluble N-terminal peptide was generated by PCR. The sense primer contained a T7 polymerase site and the coding sequences for amino acid 1-6. The antisense primer contained 18 bases corresponding to the coding region of aa33-39 plus a stop codon.

Generation of cRNAs. cRNAs were in vitro synthesized using MEGAscript RNA transcription kit (Ambion, USA) with cap analog m7G(5′)ppp(5′)G. The synthesized cRNAs were extracted with phenol/$CHCl_3$, precipitated with ammonium acetate. The identity and quantity of synthesized cRNAs were resolved by formaldehyde-containing agarose gel and UV absorbance at 260 nm.

Expression in Xenopus oocytes. Stage V-VI *Xenopus laevis* oocytes were dissected from ovarian lobes and stored in ND96 solution which contained in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4. Oocytes were injected with 50 nl containing 10 ng in vitro-transcribed cRNAs of rabbit Kv1.3 or water as control. In some experiments, 10 ng cRNA of wild-type or mutant Kv1.3 was co-injected with 10 ng cRNAs for dynamin or for a soluble N-terminal peptide. Since the cRNA for the N-terminal peptide is only 141 bases long whereas the cRNA for rabbit Kv1.3 is 1.5 kilobases, the oocytes co-injected with equivalent nanograms of cRNAs for rabbit Kv1.3 and soluble N-terminal peptide have a much higher (11-fold higher) molar concentration of cRNA for the N-terminal peptide vs cRNA for rabbit Kv1.3.

Whole cell currents were recorded as previously described (2). Briefly, a standard two-microelectrode voltage clamp (OC-725, Warner Instrument) was used to record voltage and current from *Xenopus* oocytes 2 days after cRNA injection. Oocytes were impaled with microelectrodes filled with 0.5 M KCl (resistance 0.5-5 MΩ). The bath contained in mM: 88 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2.5 NaH_2CO_3, 5 HEPES, pH 7.4. Current-voltage (I-V) relationships were obtained by applying command voltage steps and measuring the resulting membrane current. The command voltage square was held for a duration of 1 second. Measured currents were filtered at 2 kHz and then recorded and analyzed using Pulse and Pulse-fit (Heka Lambretch, F.R.G.). The peak currents at +60 mV were taken to compare the effect of mutations on K^+ channel expression.

RESULTS

The relationship between concentration of injected cRNAs and amplitude of expressed current. We created a deletion mutation D3-39 at the amino terminus of rabbit Kv1.3 by PCR. The mutation was generated in order to test if the region immediately preceding the T1 recognition domain (aa46-176) (6–8) was important for channel function. A deletion of 36 amino acids (D3-39) resulted in a 6- to 10-fold increase in whole cell currents 2 days after injection of 10 ng cRNA (Fig. 1A). The current-voltage (I-V) plots of peak currents are shown in Fig. 1B. Our previous study already demonstrated that deletion-induced elevation of K^+ current was caused by increased expression of K^+ channel protein in the plasma membrane (5). N-terminal deletion does not affect channel activity (5). Based on this, in this study we use deletion-induced change in K^+ current as a measure of expressional level of K^+ channel proteins at the plasma membrane. The peak currents at +60 mV were taken for comparison of expressional levels between wild-type and deletion constructs. For both wild-type channel and deletion mutant D3-39, the peak current at +60 mV rose as the amount of injected cRNAs increased (Fig. 1C). For wild-type channel, the peak current saturated when concentration of injected cRNA was raised above 0.5 ng/per cell. A comparable increase in cRNA concentration of mutant D3-39 did not cause saturation of peak current. At the same amount of cRNAs, mutant D3-39 always expressed currents at a higher level than that of the wild-type channel. However, we were unable to evaluate the effect of larger amount of mutant cRNA on current expression since oocytes injected with 100 ng D3-39 cRNA often had currents exceeding the capacity of the voltage clamp (100 μA).

The effect of cap analog concentration on channel expression. cRNA transcripts used for microinjection were synthesized in vitro with cap analog m7G(5′)ppp(5′)G. The cap structure in the synthesized cRNAs both functioned in the protein synthesis initiation process and also served to protect the cRNAs from degradation by intracellular nucleases. A reaction which contains high level of cap analog results in a high proportion of the transcripts being capped (12). To test whether any variation in cap reaction might significantly affect the expression, we varied the concentration of the cap analog during in vitro cRNA synthesis and then examined its effect on the levels of K^+ current expression. Figure 2 illustrated that the cRNAs synthesized without cap analog do not express K^+ current in oocytes. However, a change in concentration of cap analog from ratio of 1:4 to 1:10 (GTP to m7G(5′)ppp(5′)G) only increased the expression of K^+ current by less than 20%. We used a ratio of 1:10 for the rest of experiments when synthesizing both wild-type and mutant cRNAs to ensure a stable expression.

Site-directed mutagenesis. A deletion of 36 amino acids (D3-39) at the N-terminus of Kv1.3 channel produced an increase in expressed K^+ current. In order to precisely locate the critical amino acid residue(s) associated with the change in K^+ current, smaller deletions within this segment of 36 amino acids was generated using PCR-based mutagenesis protocols (10, 13). Figure 3C illustrated that progressive deletion at the N-terminus from amino acid 3 to 27 caused no detectable changes in whole cell currents. Further deletion to posi-

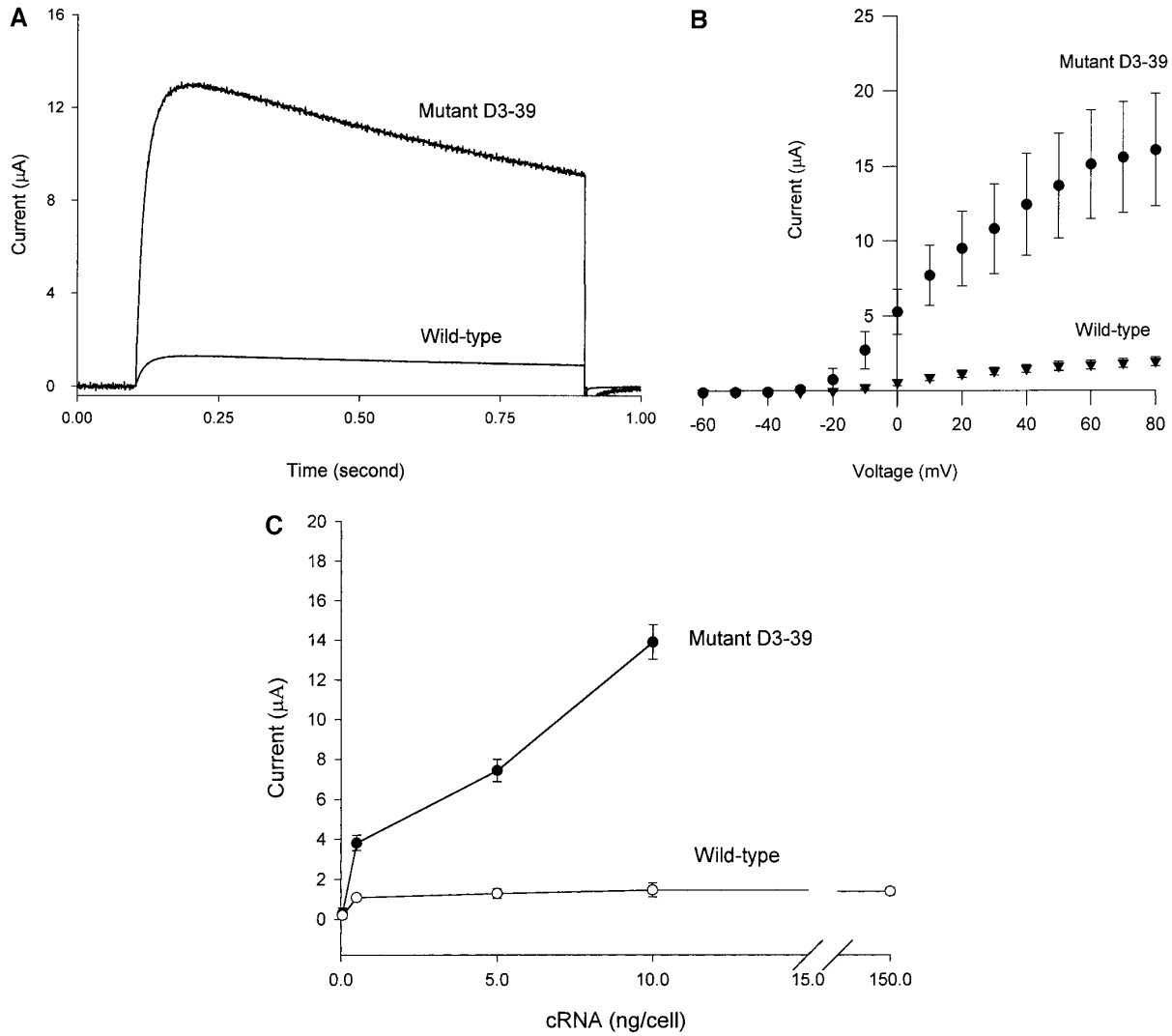

FIG. 1. Deletion mutation D3-39 expresses higher currents than wild -type Kv1.3 channel. (A) Whole cell currents for wild-type and deletion mutation D3-39. The currents were elicited by two-microelectrode voltage clamp using 1 second pulse of +60 mV from a holding potential of -80 mV with a 15 s interval to allow channels to recover from inactivation. (B) Peak current-voltage relationship for wild-type and D3-39. Each point represents means ± SE (Wild-type: $n = 18$; D3-39: $n = 30$). (C) Dependence of expressed current on cRNA concentration. 0.5 to 15 ng cRNAs were injected into *Xenopus* oocytes. The same voltage clamp protocol as 1A was used. The peak currents at +60 mV were plotted vs concentration of cRNAs injected into the oocytes. Each point represents means ± SE ($n = 6-9$).

tion 34 (D3-34) led to an amplification in the magnitude of the expressed current. Elevation in expressed current persisted in deletion mutations D3-36, D3-39, and D3-42. Deletion mutation D3-53, however, expressed currents at a lower level than that of wild-type channel. Since aa46-176 is known as T1 recognition site which mediates subunit multimerization during assembly of voltage-gated K^+ channels (6, 7), the low expression of D3-53 can be explained by destruction of T1 recognition site, which may then reduce the number of functional channel tetramers. A similar deletion at the N-terminus of mouse Kv1.3 was reported to decrease the whole cell currents (14).

From Fig. 3C, an apparent jump in the expressed K^+ current can be observed between deletion mutation D3-27 and D3-34, suggesting that the segment 28G-34P is important for regulation of channel expression. Since removal of the segment 28G-34P had no effect on current expression (Fig. 3D), some additional amino acids between aa3-27 must also be required for the regulation of K^+ current expression. To search for these additional amino acids, another series of deletion mutations were constructed which started from various positions at the N-terminal side and ended at the amino acid 34P. Figure 3D illustrated that deletion constructs D20-34 and D12-34 expressed K^+ current at a level similar to that of wild-type channel, whereas construct D6-34 expressed an amplified current at a level close

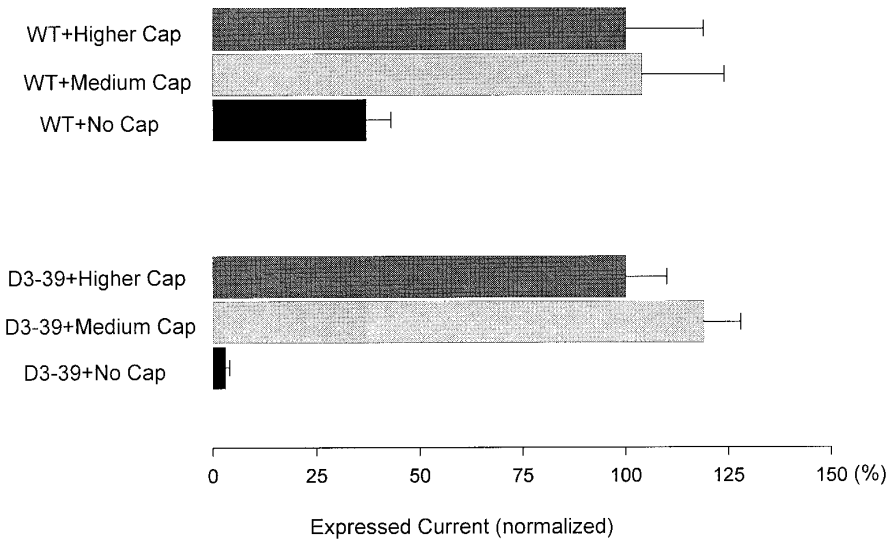

FIG. 2. The effect of cap analog concentration on Kv1.3 current expression. The cRNAs were synthesized in T7-MEGAscript reactions in which the concentration of m7G(5′)ppp(5′)G was varied from 0 (No Cap) to a ratio of 1 GTP:4 cap analog (Medium Cap) to a ratio of 1 GTP:10 cap analog (Higher Cap). The purified cRNAs were injected into *Xenopus* oocytes. The expressed currents were recorded and plotted as described in Fig. 1. The currents were normalized to percentage using the expression in High Cap condition as 100%. Each bar represents means ± SE (n = 6).

to that of construct D3-34. The shortest deletion required for the elevation of channel expression was, therefore, deduced to be D6-34.

Peptide segment aa6-34 contained 28 amino acid residues which were longer than most known consensus sequences for posttranslational modifications. Indeed, a search for consensus sequences did not reveal any obvious consensus sequence for posttranslational modifications (phosphorylation, glycosylation, etc.). Inspection of the primary amino acid sequence, however, identified 8 charged amino acid residues in aa6-34. High percentage of charged residues implicated a possible involvement of charged residues in the regulation of channel expression. It is possible that the elevation of K^+ current caused by deletion mutations is associated with removal of charged residues. A support to this hypothesis was provided by the result that the effective deletion to achieve the elevation in K^+ current should include both ends of segment aa6-34, and coincidentally the charged residues were mostly distributed at both ends of the segment (Fig. 3A). An amino acid substitution experiment was performed to replaces two charged residues (31R, 33E). Construct D3-27 failed to elevate the expression of K^+ current whereas construct D3-27* which had two less residues (31R, 33E) could increase the expression to the level of D3-39 (Figs. 3A and 3C). Taken together, these results suggested an important role of charged residues in the regulation of channel expression. Figure 3B illustrated the hydropathic profile of the N-terminal region by Kyte-Doolittle method. An overwhelming hydrophilicity in the segment of aa6-34 was consistent with high percentage of charged residues in the region.

Effect of a mutated-dynamin on expression of Kv1.3. Previous data demonstrated that the elevation of K^+ current caused by the N-terminal deletion of rabbit Kv1.3 was associated with the increased number of channel proteins expressed in the plasma membrane of *Xenopus* oocyte (5). The elevation of expression of channel proteins could result from increased translation, stimulated intracellular trafficking of synthesized proteins toward the plasma membrane, or decreased endocytotic retrieval. We used a mutated dynamin (K44A) to block endocytotic process in order to examine the possible involvement of endocytosis. This construct was successfully used to block endocytosis by other research groups (11). The mutated dynamin caused a slight increase in expressed K^+ current (121 ± 4%, Table 1) when co-injected with wild-type Kv1.3 whereas it had no statistically significant effect on the expression of the mutant D3-39 (Table 1). As a control, the effect of wild-type dynamin was examined. The wild-type dynamin had no effect on the expression of the wild-type Kv1.3 or mutant D3-39 (Table 1).

Effect of excessive amount of soluble N-terminal peptide. We examined the effect of excessive amount of soluble N-terminal peptide aa1-39 on K^+ current expression. The cRNA of the wild-type Kv1.3 was injected into *Xenopus* oocytes together with the expressive amount of cRNA for soluble N-terminal peptide aa1-39. The oocytes co-injected with cRNAs at a ratio of 1:20 (Kv1.3:N-terminal peptide) expressed a lower level of current (72 ± 6%, Table 1) than those oocytes injected with Kv1.3 alone. The effect of the N-terminal peptide was specific to Kv1.3 channel since injection of

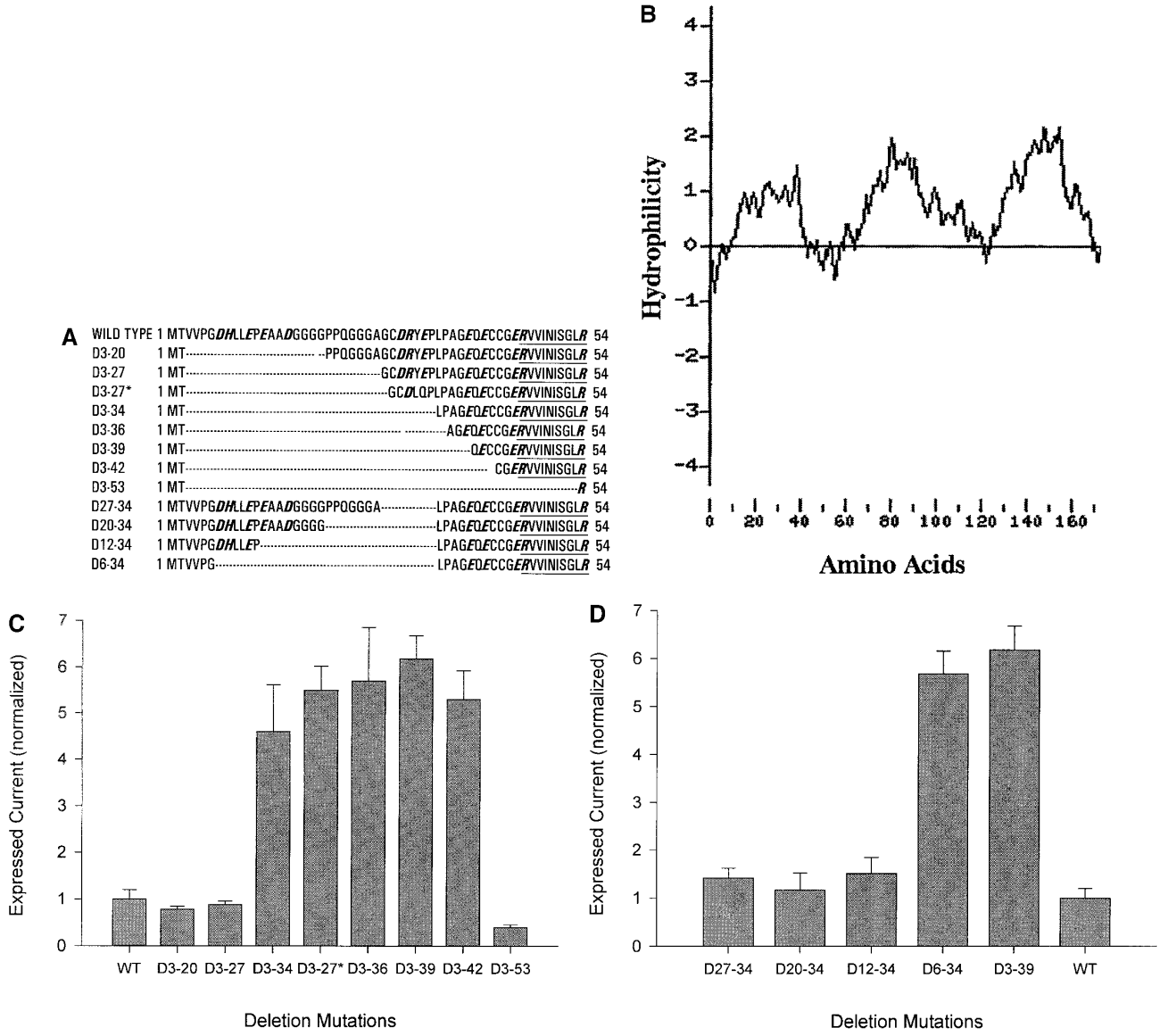

FIG. 3. The charged residues at N-terminus may regulate the channel expression. (A) denoted the region of N terminus that was mutated. Charged residues are in bold and italics. T1 domain is underlined. (B) illustrated hydropathic profile of the N-terminal region of rabbit Kv1.3 by Kyte-Doolittle method with a window length of 17. (C) showed the effect of progressive deletion started from position 3 in the direction of the amino terminal side towards the carboxyl terminal side. (D) presented the effect of progressive deletion started from position 34 in the direction of the carboxyl terminal side towards the amino terminal side. The expressed currents were normalized to percentage using the expression of D3-39 as 100%. Each bar represents means ± SE (n = 6–10).

the same amount of cRNA for N-terminal peptide does not affect the expression of another K^+ channel, Kv1.5 channel (101 ± 9%, peak current at +60 mV, n = 5). The fact that the expression of Kv1.5 channel was not reduced by soluble N-terminal peptide indicated that translational apparatus in oocytes was not saturated by the amount of N-terminal peptide expressed. Therefore, the decreased expression of Kv1.3 caused by excessive amount of the N-terminal peptide could not be attributed to a saturation of the translational apparatus by soluble N-terminal peptide.

DISCUSSION

The N-terminal region of Kv1.3 contains a signal sequence that regulates the expression of K^+ channel proteins in the plasma membrane at least in rabbit Kv1.3 (5). A deletion of 36 amino acids (D3-39) resulted in a 6- to 10-fold increase in the amplitude of expressed K^+ current (Fig. 1A). To search for the critical amino acids responsible for the change in K^+ current, we made a variety of smaller deletions within the peptide segment aa3-39 and examined the effect of these deletions on

TABLE 1

Effect of a Mutated Dynamin (K44A) or N-Terminal Peptide on the Expression Level of Kv1.3

	Control (%)	Co-injected with mutated dynamin (%)	Co-injected with wild-type dynamin (%)	Co-injected with N-terminal peptide (%)
Wild-type	100 ± 6 (7)	121 ± 4 (7)*	104 ± 18 (7)	72 ± 6 (13)*
Mutant	100 ± 14 (13)	109 ± 9 (15)	92 ± 12 (15)	—

Note. Mean ± SE (*n*).
* $P < 0.05$ vs control of wild-type.

the expressed K^+ current. The results in Fig. 3 indicate that the signal sequence peptide is located in segment aa6-34. There is a certain length requirement for active function of this signal sequence. The shortest deletion required for the elevation of K^+ current is D6-34. Smaller deletions which did not include either end of this peptide segment were not be able to elevate the expression of K^+ channel. Since the functional signal sequence has to include both ends of the peptide segment aa6-34 where charged residues are densely distributed (Figs. 3A, 3C, and 3D), it is conceivable that this N-terminal signal sequence is related to charge or its associated hydrophilicity. Amino acids substitution experiment was carried out to confirm the importance of charged residues. Deletion construct D3-27 fails to elevate the expression of K^+ current whereas construct D3-27* which has replacement of two charged residues (31R, 33E) effectively raises the amplitude of expressed current close to the level of D3-39 (Figs. 3A and 3C). This result further suggests that the N-terminal signal sequence may be related to charge or its associated hydrophilicity.

The elevation of K^+ current by N-terminal deletion of Kv1.3 was associated with increased expression of K^+ channel protein in the plasma membrane (5). Charged residues may affect K^+ channel expression at the plasma membrane by altering topological orientation and causing segregation of K^+ channel proteins into different cellular compartments (15–19). Peptide segment aa6-34 which contains a signal sequence for expressional regulation carries 8 charged residues. Removal of these charged residues may disturb the charge balance and/or produce a conformational change in the N-terminus, causing a change in the intracellular sorting of the channel proteins. A related topic is that the peptide segment aa6-34 is highly hydrophilic (Fig. 3B). Hydrophilicity in the N-terminus of membrane proteins has been reported to affect the folding states of the N-terminus, resulting in an alteration in membrane translocation and a change in intracellular sorting of membrane proteins (17, 19, 20). Removal of the hydrophilic domain aa6-34 may produce a conformational change at the N-terminus and therefore affect intracellular sorting of the channel proteins.

Deletion-induced increase of K^+ channel proteins in the plasma membrane could be caused by increased translation, stimulated intracellular trafficking of synthesized proteins toward the plasma membrane, or decreased endocytotic retrieval of membrane proteins. Position 32Y-35L contains a putative tyrosine-based sorting motif which may facilitate the endocytotic internalization of membrane proteins (21). Removal of this sorting motif could block the endocytotic retrieval, thus causing an elevation in the amount of channel proteins expressed in the plasma membrane. To test the role of endocytosis, we used a mutated dynamin to block the endocytosis and then examined its effect on the expression of K^+ current. Dynamin is a GTPase known to provide a critical accessory function in clathrin-dependent endocytosis (21–23). By complexing around the neck of invaginated coated pits, it may provide a mechanochemical force to accomplish the final step in the budding process during the endocytosis. Mutated dynamin was shown to inhibit the clathrin-dependent endocytosis (11, 24). Our data indicate that the blockage of endocytotic process by a mutated dynamin K44A only slightly increases the expression of K^+ current (Table 1). Therefore, a 6- to 10-fold elevation of K^+ current caused by the deletion mutation D3-39 can not be accounted by the blockage of endocytotic pathways. We further explored the role of endocytosis by co-injecting the cRNA for the wild-type Kv1.3 together with excessive amount of cRNA for soluble N-terminal peptide aa1-39. If the N-terminal region (aa1-39) of Kv1.3 contains a sorting motif for endocytotic retrieval, excessive amount of soluble N-terminal peptide aa1-39 may saturate or at least compete with the endocytotic retrieving apparatus, causing a decrease in the rate of endocytotic retrieval and therefore an increase in the number of K^+ channel proteins expressed in the plasma membrane. However, our data clearly indicate that the excessive amount of soluble N-terminal peptide does not raise the amplitude of K^+ current (72% vs control at 100%). Taken together, our results suggest that the N-terminal signal sequence may not act through endocytotic pathway and the putative tyrosine-based sorting motif 32Y-35L is not a functional motif. It is more likely that the N-terminal signal sequence may regulate the processes which lead to the channel insertion into the plasma membrane. These

include protein synthesis and delivery of synthesized proteins to the plasma membrane.

Elevation of Kv1.3 current is required for activation and proliferation of T lymphocytes. K^+ current elevation during T cell activation shares a striking similarity to the K^+ current amplification caused by N-terminal deletion (D3-39). The kinetics of K^+ current increase is similar in both cases (4, 5), and translational/posttranslational modification(s) on the channel may result in the increased K^+ current in both situations (5, 25, 26). Based on the similarity in the mechanism of K^+ current elevation in deletion mutation D3-39 and T lymphocyte activation, it is tempting to suggest a regulatory role for the amino terminus of Kv1.3 in the elevation of K^+ current during the T lymphocyte activation process. It is possible that a conformational change at the N-terminus of Kv1.3 may lead to an increased rate of K^+ channel insertion into the plasma membrane, resulting in an elevation of K^+ current and eventually leading to the activation and proliferation of T lymphocytes.

In conclusion, we have found that the N-terminus of rabbit Kv1.3 contains a signal sequence which regulates the expression of channel proteins in the plasma membrane. The regulatory role of the N-terminus may be determined by charge or its associated hydrophilicity. It is likely that the N-terminal signal sequence controls some processes which mediate the channel insertion into the plasma membrane.

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