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A Central Role for the T1 Domain in Voltage-gated Potassium Channel Formation and Function*

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To interpret the recent atomic structures of the Kv (voltage-dependent potassium) channel T1 domain in a functional context, we must understand both how the T1 domain is integrated into the full-length functional channel protein and what functional roles the T1 domain governs. The T1 domain clearly plays a role in restricting Kv channel subunit heteromultimerization. However, the importance of T1 tetramerization for the assembly and retention of quarternary structure within full-length channels has remained controversial. Here we describe a set of mutations that disrupt both T1 assembly and the formation of functional channels and show that these mutations produce elevated levels of the subunit monomer that becomes subject to degradation within the cell. In addition, our experiments reveal that the T1 domain lends stability to the full-length channel structure, because channels lacking the T1 containing N terminus are more easily denatured to monomers. The integration of the T1 domain ultrastructure into the full-length channel was probed by proteolytic mapping with immobilized trypsin. Trypsin cleavage yields an N-terminal fragment that is further digested to a tetrameric domain, which remains reactive with antisera to T1, and that is similar in size to the T1 domain used for crystallographic studies. The trypsin-sensitive linkages retaining the T1 domain are cleaved somewhat slowly over hours. Therefore, they seem to be intermediate in trypsin resistance between the rapidly cleaved extracellular linker between the first and second transmembrane domains, and the highly resistant T1 core, and are likely to be partially structured or contain dynamic structure. Our experiments suggest that tetrameric atomic models obtained for the T1 domain do reflect a structure that the T1 domain sequence forms early in channel assembly to drive subunit protein tetramerization and that this structure is retained as an integrated stabilizing structural element within the full-length functional channel.

The structural elements of potassium channels have begun to be characterized in atomic detail, allowing much increased sophistication in our understanding of their mechanism of action and biological function. For voltage-dependent potassium (Kv)[1] channels, the structure of the highly conserved cytoplasmic N-terminal T1 domain has been determined as a rotationally symmetric tetramer from three different Kv channels and in complex with an auxiliary β-subunit protein (1–4). Because the T1 domain structures are determined from isolated soluble protein domains, questions arise as to the relevance of the determined structures to the ultrastructure of the full-length Kv channel and in how the tetrameric domain is integrated into the remainder of the channel. Recent published studies have suggested that the T1 structure within the channel is likely to be very similar to the tetrameric structure of the isolated domain (3, 5–7). However, crystallography also has shown that the T1 domain can adopt several different conformations within the channel that may be involved in regulating channel gating properties (3, 4, 6). Specifically, there is conformational variability in the C-terminal region that is anticipated to be against the membrane (4, 6). Furthermore, the T1 domain has been found to adopt a three-dimensional fold, called the BTB/POZ fold, that is observed in other protein complexes (8–12). Interestingly, other BTB/POZ subunits often exist in oligomeric association as monomeric or dimeric subunits, and there are multiple binding faces for other subunit proteins found within this fold as well. This particular domain then affords many possible binding interactions to itself, to other domains of the full-length channel, or to other proteins. Clearly, then, it becomes important to assess the ultrastructure of the T1 domain within the intact channel to formulate models for channel mechanism and biological activity.

The T1 domain was identified originally as an important site for regulating intersubunit interactions during channel assembly, such that only a restricted number of heterotetramers are made from the many possible Kv channel subunit proteins that are synthesized within a single cell (5, 13–22). Nevertheless, several groups have reported the functional expression of Kv channels after the deletion of the T1 domain in channel overexpression systems (13, 15, 23), in contrast to other reports that T1 deletion precludes channel assembly (5, 20, 22, 24). The fact that channels can form without a T1 domain might be expected, because the membrane itself can serve to nucleate secondary structure into incorporated peptides via the hydrophobic effect (25), and the transmembrane core of the Kv channel is shared with a large number of homologous and tetrameric channel proteins that lack a T1 domain (26, 27). However, it does call into question what T1-T1 interactions might add to Kv channel formation and the resultant ultrastructure and whether these conformations might be a dynamic aspect of channel gating.

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[1] The abbreviations used are: Kv, voltage-dependent potassium; BTB, broad complex, tramtrack, bric-a-brac; S1, first transmembrane segment, S2, second transmembrane segment; SEC, size exclusion chromatography; GFP, green fluorescent protein; EGFP, enhanced GFP; CHO, Chinese hamster ovary; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AD, activation domain; BD, DNA-binding domain; POZ, pox virus and zinc finger.

We know from electrophysiological data (3, 4) that there is a relationship between T1 conformation and gating, even if we do not know the exact details of how the T1 domain integrates into the intact channel ultrastructure, such as the number and nature of its contacts with other channel elements. Point mutations within the T1 domain of two different Shaker channels alter channel electrophysiology, suggesting that there is a very tight energetic and therefore conformational link between T1 and the channel gating elements. Based on the above electrophysiology and the rotational symmetry of the tetrameric T1, we suggest that the T1 domain lies in alignment with the pore and gating elements as has been proposed (3, 4, 6). This suggests that the T1 domain C terminus abuts S4 and possibly interacts with S6 and the channel C terminus, but this has not yet been demonstrated.

Recent data from the Miller laboratory (7) have shown that the atomic structure of the isolated tetramer can be used to correctly predict cross-linking pairs of amino acids in the full-length channel, indicating that the crystal structure of the T1 domain does approximate the ultrastructure in this portion of the full-length channel. Additionally, they suggest that the T1 domain is a "hanging gondola" tethered to the membranous channel portions by "cables" of unknown structural characteristics. Unfortunately, relatively little is known about how the T1 domain is integrated into the remainder of the channel. It is expected that the T1-S1 linkage lies near the outer edge of the channel, because S1 appears to be a lipid-facing transmembrane segment (28–30), but structural details of the linkage between the T1 domain and the first transmembrane segment, S1, are lacking because of solubility problems encountered with crystallization attempts on this construct.

In a recent article from the Jan laboratory (31), a completely distinct tetrameric motif of roughly the same dimensions as the T1 domain was found to provide a scaffolding platform for the efficient and high yield formation of a surface channel even though the permuted channel itself had altered kinetic and gating properties. Based on similarities in the electron micrographs of Kv channels and the nicotinic receptor (32), it has been proposed that an aqueous gap exists between the T1 domain and the transmembrane domains in the open channel (33, 34) and that the linkage here corresponds to the cables of the hanging gondola model (7). If it is the primary link from T1 to the membrane integrated channel and itself contains little structure or it folds independently of the T1 domain, this linker may explain why the Jan laboratory successfully substituted a completely separate tetrameric motif for T1.

We have taken a mutational approach toward the study of T1 subunit interactions and their role in the structure and stability of the intact channel. We have generated a set of point mutations that affect subunit interactions in T1 and subsequently disrupt tetramer formation. These mutations were generated from a random screen, yet the mutated residues are the very same amino acids that are conserved among either all Kv channels or the Shaker subfamily. We next examined the ability of full length Kv subunit proteins to form channels when the T1 region contained these same mutations and found disruptions in tetrameric channel assembly, as would be expected if T1 tetramer formation drives the assembly and cell surface expression of the intact channel. Within the crystal structure of T1, these mutated amino acids were found either at subunit interfaces or at critical locations for subunit folding, and thus, the perturbations in channel assembly provide independent confirmation of the absolute requirement for correct T1 folding and assembly to occur in the intact channel.

We then present further biochemical analysis of these mutants and the native intact channel to examine the role of T1 in the dynamic regulation of channel assembly and in the ultrastructure of the intact channel.

MATERIALS AND METHODS

Yeast Two-hybrid Identification and Analysis of T1 Mutations—Yeast two-hybrid analysis was conducted according to the method of Fields and Song (35), using a Gal4 two-hybrid phagemid vector kit (Stratagene), and the manufacturer's instructions for the quantitative determination of β-galactosidase expression, with *o*-nitrophenyl-β-D-galactopyranoside as a colorimetric substrate. Region-specific random mutagenesis was performed using randomly mutagenizing oligonucleotides of ~90 bases (Ref. 36). (Oligonucleotides are synthesized to a specific region with doped bases, in which each base is doped at the 0.15% level with the other three bases. This doping creates a low probability of misincorporation at every position along the oligonucleotide. If each base has an equal probability of incorporation, then the calculated mutation rate is low enough to produce most full-length oligonucleotides with 0–2 random point mutations per oligonucleotide.) Mutagenizing oligonucleotides were used in primer extension reactions, made double-stranded, and cassette-cloned into Gal4-AD-T1. Libraries of mutant T1 domains were made by transforming the ligations into bacteria and preparing the DNA from >98% colonies in the plate of transformants, taking care to avoid disparity in colony growth. These cDNA libraries were then transformed into Gal4-BD-T1 yeast and plated at a low density to allow clear growth of single colonies. T1 assembly mutants were identified by the inability of a yeast colony containing both vectors to express β-galactosidase. The mutated T1 cassettes, which resulted in white colonies as Gal4-AD-T1 mutants, were swapped into the Gal4-BD-T1 vector and retested against wild-type Gal4-AD-T1. For most mutations, the results were very similar when the mutation was placed in either the AD or BD fusion construct. Mutations were identified by DNA sequencing of the entire cassette. Further characterization by *in vitro* translation of mutant T1 domains and identification of monomer and tetrameric fractions by size exclusion chromatography (SEC) was performed as described previously (21). For making the full-length constructs, mutant T1 domains were cassette-cloned into the Aplysia Shaker Kv1.1 coding sequence either with or without an EGFP tag made by in-frame cloning of EGFP after residue 477 of the aKv1.1 C terminus. To enhance characterization in physiology studies, the first four N-terminal amino acids of aKv1.1 were removed to reduce *N*-type inactivation.

For the analysis of monomer and tetramer fractions of T1 mutant full-length channels proteins were transfected into CHO cells, and membranes were isolated as described below and then solubilized in 1% CHAPS either with or without added SDS at 1%. SEC-FPLC separation was performed with an Amersham Pharmacia Biotech FPLC over a Superose-6 column with added CHAPS to 0.5%.

Biophysical Analysis of Normal and T1 Mutant Channels—Full-length constructs were expressed in *Xenopus* oocytes by either microinjection of cytomegalovirus promoter cDNA expression constructs into the nucleus of *Xenopus* oocytes or injection of cRNA made *in vitro* with T7 RNA polymerase. For each batch of oocytes, a control wild-type construct expression was used to determine the expected amount of channel expression for a given length of time. Recordings were performed from 1 to 3 days after injection as described previously (6). Expression levels were compared by measuring peak current levels in response to a voltage pulse to +60 mV for 100–200 ms.

Microscopy of T1 Point Mutations in Transfected COS7 Cells—All tissue culture supplies were purchased from Life Technologies, Inc. except for serum that was purchased from Hyclone, Inc. The microscopic analysis of cells transfected with Kv1.1-EGFP and its mutants was performed on a Zeiss LSM-510 confocal microscope, using the recommended settings for EGFP detection, with parallel capturing of the optical image. Serial sections were taken in 0.8-micron steps starting at the cell–coverslip interface using an ×63 oil-immersion objective lens. Representative sections are shown. COS7 cells plated on glass coverslips were transfected with Fugene™ (Roche Molecular Biochemicals) as recommended by the manufacturer using the same cDNA constructions that were tested in oocyte expression studies. At 24–30 h post-transfection, the cells were washed with ice-cold PBS, fixed in 3% formaldehyde (EMS, Ft. Washington, PA) in PBS/0.15 mM Ca^{2+} for 30 min, washed again with ice-cold PBS, and mounted with VectaShield™ (Vector Laboratories, Burlingame, CA). Colocalization experiments to demonstrate that the monomeric subunits are retained in the endoplasmic reticulum were conducted using fluorescence immunocytochemistry with antisera to an endoplasmic reticulum protein standard, calnexin, and a secondary antibody tagged with rhodamine (both antisera

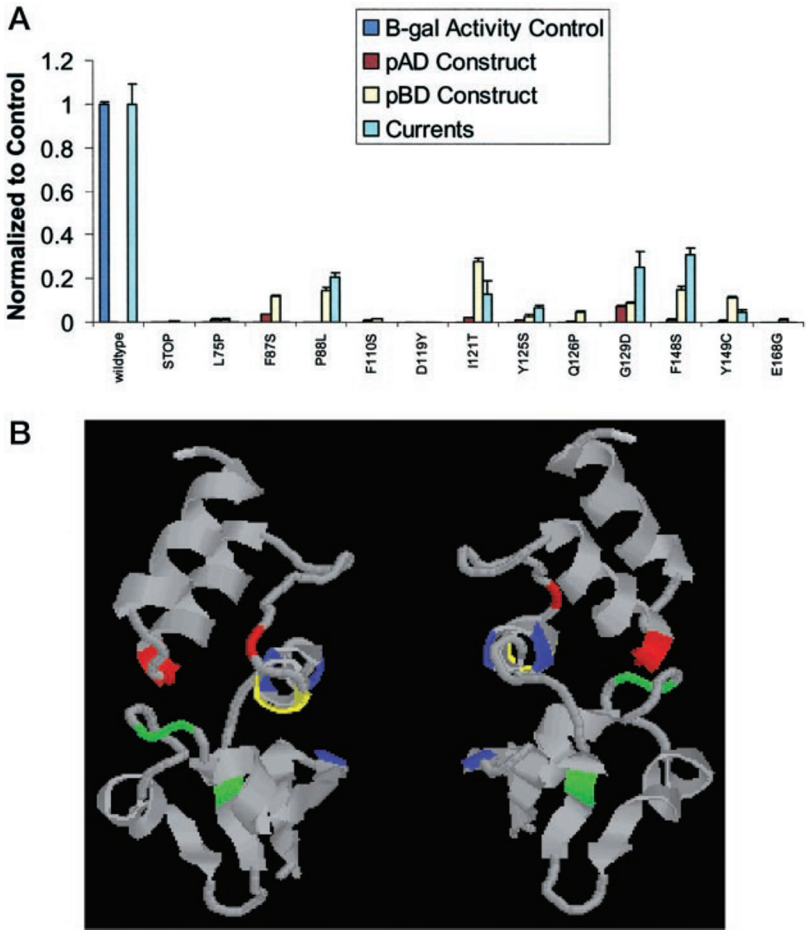

FIG. 1. **T1 assembly mutations disrupt functional channel expression.** *A*, mutations disrupting T1 domain interactions were assayed by the loss of expressed β-galactosidase activity. T1 mutant constructs were tested as fusions with either Gal4-AD (*pAD*) or Gal4-BD (*pBD*) against a wild-type T1 domain in the complementary Gal4 vector. β-galactosidase activity was determined by *o*-nitrophenyl-3-D-galactoside cleavage to produce *o*-nitrophenol, which was measured by spectroscopy at 500 nm. β-galactosidase activity with the wild-type T1 domain in both constructs was used as 100% control, and the activity of all mutants was normalized to the activity of wild type. Whole-cell recordings of aKv1.1 channels containing mutations within the T1 domain of the full-length channel expressed in *Xenopus* oocytes. The results shown here are from nuclear injections with aKv1.1 cDNAs possessing a C-terminal EGFP tag. Similar results were obtained with either cDNA or cRNA injections and with or without a C-terminal EGFP tag. The T1 mutants all show a loss of functional channel expression that is similar to the measured loss of interaction in yeast two-hybrid measurements. These results suggest that the loss of functional channel expression is related to the magnitude of the T1 assembly deficit as measured in yeast. *B*, the structural diagram of the T1 domain and mutant positions is highlighted. Mutated positions are clustered as follows: *blue*, subunit interface mutants; *green*, layer 1 packing mutants; *yellow*, bridge helix mutants; and *red*, layer 3 packing mutants. The T1 domain has been shown to fold into a newly described but now quite common three-layer scaffold motif termed the BTB/poxvirus and zinc finger domain. BTB domains are found in nature as monomers either singly or in multisubunit complexes, homo- or heterodimers, and tetramers. All known Kv channel T1 domains follow this fold. Notable examples of other BTB domains are the PLZF (promyelocytic leukemia zinc finger) domain (9), Elongin C of the von Hippel Laundau tumor suppressor complex (10), and Skp C of the Skp1-Skp2 ubiquitin ligase complex (11).

from Santa Cruz Biotechnology, Inc.). Briefly, fixed cells were permeabilized with a CHAPS wash of 0.5% CHAPS in PBS for 30 min, blocked with normal donkey serum (Cappel Laboratories) at a dilution of 1:10 in PBS, reacted with the primary antiserum at a dilution of 1:100 for 30 min, washed with PBS (three changes over 30 min), and finally reacted with the secondary antiserum at a dilution of 1:100 prior to microscopy.

Analysis of the T1 Domain within the Aplysia Kv1.1 Channel—Membrane preparations of aKv1.1 (37) from a stable CHO-derived cell line expressing a C-terminal EGFP-tagged aKv1.1 channel were made by low ionic strength lysis of the cells (1 ml of 50 mM Tris-HCl and 1 mM EDTA, pH 7.5 in a 100-mm dish of cells at >85% confluency with the following protease inhibitors added: 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and a mixture of EDTA-free protease inhibitors from Roche Molecular Biochemicals) at 0 °C, followed by brief sonication, a low speed spin of 1500 rpm for 2 min to remove organelles, and a high speed spin of 60,000 rpm for 20 min to pellet the membranes. The pellet was resuspended in PBS, sonicated briefly to obtain a uniform opalescent suspension, and subjected to trypsin digestion. Trypsin immobilized onto agarose beads in a 1:1 suspension with H_2O added at a ratio of 1:20 (ml of membrane preparation:beads), and the mixture was rotated for 16 h at room temperature. The digested Kv1.1 preparation was passed through a 0.2-micron filter to remove trypsin and membrane-associated proteins before separation on a Superose-12 SEC-FPLC column (Amersham Pharmacia Biotech) run in 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, and 150 mM NaCl. Fractions were acetone-precipitated and run on 15% SDS-PAGE gels, and the locations of proteins were determined by Western blotting (21).

For chemical cross-linking, the released trypsin-resistant tetramer was isolated as described above and compared with the T1 domain purified from bacterial expression. All cross-linking reagents were from Pierce. Tested cross-linkers included *N*-α-[maleimidoacetoxy]succinimide ester, bismaleimidoethane, disuccinimidyl glutarate, *N*-succinimidyl iodoacetate. Cross-linking was carried out for 30 min at room temperature in the FPLC buffer, using 1 mM cross-linking reagent, and then stopped using L-cysteine at 2.5 mM. Proteins were separated on 15% SDS-PAGE gels (38) to determine the extent of cross-linking.

RESULTS

Identification of T1 Assembly Mutants—We first sought to examine how the formation of the functional channel is affected by T1 domain point mutations that demonstrate altered intersubunit T1 domain interactions. To address this question, we first identified point mutations within the T1 domain that disrupt the ability of the domain to tetramerize. We then determined whether these point mutations disrupt intact Kv

TABLE I
Residues important for tetramer formation in Kv1.1 T1 domain generated from a yeast two-hybrid screen used to randomly generate inhibitory mutations

Residue	Location, T1 structure	Interpretation for loss of tetramer formation
1 L75P	Layer 1, N terminus, β-strand 2	Interrupts β1→β2 loop to β2 strand interaction at subunit interface
2 F87S	Layer 1, interhelical loop α_1–α_2 conserved among all Kv	Destabilizes intradomain packing between layers 1 and 3
3 P88L	Layer 1, interhelical loop α_1–α_2 conserved among all Kv1.*x*	Change polypeptide chain angle, destabilizes intradomain packing between layers 1 and 3
4 F110S	Layer 1, C terminus, β-strand 4 conserved among all Kv	Destabilizes intradomain packing within layer 1
5 D119Y	Layer 2, mid-helix, α_3 conserved among all Kv1.*x* and adjacent to F118, which is conserved among all Kv	Interrupts polar subunit interface between Asp-119, Arg-115′, Gln-126, and Ser-73′
6 I121T	Layer 2, mid-helix, α_3 conserved among all Kv	Interrupts polar subunit interface between Asp-119, Arg-115′, Gln-126, and Ser-73′
7 Y125S	Layer 2, C terminus, α_3 conserved among all Kv	Destabilizes intradomain packing between layers 1 and 3 and helices 1 and 3, respectively
8 Q126P	Layer 2->3, variable residue sandwiched between Y125 above and S127, G128 which also are conserved among all Kv	Change polypeptide chain direction, destabilizes intradomain packing between layers 1 and 3, helices 1 and 3 respectively, possibly α_4 of layer 3 and its adjacent structures
9 G129D	Layer 3, loop conserved among all Kv1.*x*	Destabilizes intradomain packing between layers 1 and 3 and helices 1 and 3, respectively
10 F148S	Layer 3, C terminus, α_4 conserved among all Kv	Destabilizes intradomain packing between layers 1 and 3
11 Y149C	Layer 3, C terminus, α_4 conserved among all Kv	Destabilizes intradomain packing between layers 1 and 3
12 E186G	C-terminal to T1, N-terminal to S1 estimated to begin at amino acid 197. Region of ambiguous structure, possibly sheet	Unknown

channel assembly and the formation of functional channels. To rapidly characterize a large number of such point mutations, we performed a genetic screen for T1 assembly mutations using the yeast two-hybrid system. We first confirmed that the natural specificities for homomeric and heteromeric T1 assembly were reproduced in the standard yeast two-hybrid screen, as previously described (16–19). To identify point mutations of the Shaker T1 domain that disrupt assembly, we created a library of T1 domain mutations, using a randomly doped oligonucleotide-based approach (36), fused to Gal4-AD. We then cotransfected this library with a wild-type Gal4-BD-T1 fusion construct and tested it for activation of the LacZ reporter gene using X-gal. White colonies, which are indicative of a failure of the T1 domains to interact in these yeast, were characterized to determine the molecular basis for the deficit. The mutant Gal4-AD-T1 domain clones were isolated and sequenced, and their phenotype was confirmed by retransformation, transfer of the deficiency to the Gal4-BD-T1 construct by subcloning of the mutant T1 domain sequences, and failure of the mutant T1 domains to tetramerize after *in vitro* translation (5). In addition to frameshift mutations and stop codons, 12 different missense point mutations that satisfied these criteria were identified (see Fig. 1*A*). These mutations are distributed throughout the T1 domain from residues 75–168 and are found to encode a variety of changes to conserved amino acids (Fig. 1*B*). Based on the T1 structure, these mutations likely produce either steric blocks to subunit tetramerization or alter the packing between layers of secondary structure within the monomer (Table I).

Disruption of Functional Channel Formation by T1 Assembly Mutations—To determine whether point mutations that disrupt T1 domain assembly also disrupt full-length channel formation, we subcloned the mutational cassettes into full-length channel constructs with or without a C-terminal EGFP tag and expressed the channels in *Xenopus* oocytes (Fig. 1*A*). Functional recordings show that all the mutations produce severe reductions in expressed currents ranging from absent current to a current that was <30% of the wild-type current, and in general the extent of reduction in the current correlated with the extent of the reduction in β-galactosidase activity previously measured in the yeast two-hybrid experiments.

Disruption of Normal Channel Assembly and Processing by T1 Domain Mutations—To directly determine the relationship between functional channel formation and a deficit in tetramer formation, we measured the monomeric and tetrameric fractions of channel subunit proteins on Western blots after separation by SEC-FPLC. The results clearly show that the T1 domain point mutations result in a dramatic shift of the tetramer/monomer distribution toward monomer. In wild-type channel preparations, the monomeric fraction represents an estimated 10% or less of the total immunoreactive material compared with the point mutants in which the monomer is typically >50% of the total channel protein. When the protein level is very low, there is a loss of post-translational processing of the glycosylated protein, normally seen as a smearing of the tetrameric protein into higher molecular masses (22, 39) (for example, as shown for the mutants Y125S and Y149C in Fig. 2). In addition, we noted that the total amount of immunoreactivity present decreased in relationship to an increase in the monomeric subunit observed in mutants such as Y149C and Y125C after transfection of the same amount of cDNA (see Fig. 2). This relationship suggests that the mutant proteins are less stable in these cells and that it is the monomer that is selectively degraded.

We also examined the subcellular localization of C-terminal EGFP-tagged wild-type and T1 mutant subunit proteins. In general, the distributions of the proteins are similar in both cases, being limited to cellular membrane compartments as would be expected for channels that contain the wild-type sequence for the six-transmembrane domains. However, the total amount of fluorescence in the wild-type transfected cells was always higher than that seen with T1 assembly point mutants and was distributed more evenly throughout the cell. In comparison, the fluorescence intensity for channels with T1 assembly mutations is more concentrated in perinuclear compartments (Fig. 3). This finding is consistent with a retention of monomeric subunits within the endoplasmic reticulum, as confirmed by colocalization of the channel with the endoplasmic reticulum marker (calnexin).

Subunit Protein Lacking the T1 Epitope—Interestingly, in Western blots of the wild-type construct with a C-terminal

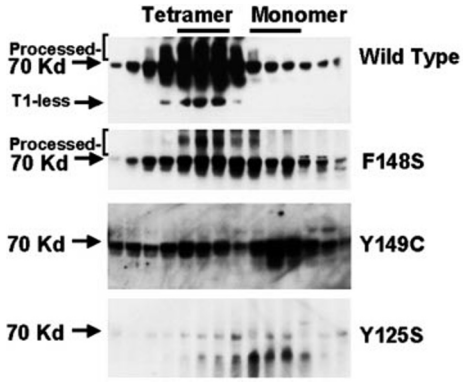

FIG. 2. **T1 mutations increase the level of monomeric protein and destabilize subunit proteins with the cell.** The data shown are SEC-FPLC separations on full-length channels containing point mutants within the T1 domain sequence to resolve tetrameric and monomeric channel populations. Membrane preparations from CHO cells transfected with aKv1.1-EGFP constructs containing T1 point mutations were solubilized in 1% CHAPS and fractionated by size exclusion chromatography. CHO cells were used for these experiments for the abundant protein expression we see in this cell line and to enable comparison to a stable cell line generated within this laboratory that we use routinely for aKv1.1-EGFP expression (see Figs. 4 and 5). (Please note that the *wild type lane* is from cells transfected with wild-type channel simultaneously and identically to the mutant channel contructions.) Fractions containing channel proteins were obtained from 5×10^6 cells transfected with 1 μg of DNA in each case. The Kv1.1 bands were identified by Western blotting using either anti-T1 or anti-EGFP antisera; the data shown are with anti-EGFP antisera. The wild-type T1 domain constructs show most of the protein in the tetrameric fractions with considerable smearing of the protein produced by channel glycosylation to varying degrees. Note the presence of a lower molecular mass band in the tetrameric fractions; this is channel material that has had the T1 domain proteolytically removed within the cells (see "Results" and Fig. 4). We first see protein loss overall and a distribution that is altered toward monomer but includes protein still found as glycolytically processed material in the tetrameric fractions. However, the overall immunoreactivity is less, and a greater proportion of the protein is found in monomeric fractions. If the overall protein level is very low, then we see additional effects other than protein loss alone; there is a complete loss of glycosylation-processed protein in the tetrameric fractions, and degradation products are seen in the monomeric fractions. *Kd*, kilodaltons.

EGFP tag, we often can detect traces of a lower molecular mass polypeptide that remains reactive to antibodies that recognize the channel C terminus but has lost reactivity with the anti-T1 domain antibody (see Fig. 2, *Wild Type*, and Fig. 4*A*). The C-terminal epitopes were 1) a peptide corresponding to residues 429–464 of the sixth transmembrane segment and a portion of the C-terminal domain, and 2) GFP. Importantly, there is no background reactivity to either of these antisera in nontransfected cells, as shown by the representative blank lanes in Fig. 4*A*. We solubilized the channel mixture under different conditions to compare the characteristics of this T1-less channel to the full-length channel tetramer. The results show that after the removal of the N terminus, the truncated channels remain in the tetrameric fractions and can be solubilized with 1% CHAPS, similar to full-length channels (Figs. 2 and 4*B*). However, solubilization under more stringent denaturing conditions such as 0.5–1% SDS (Fig 4*C*) produces a selective dissociation of the shorter channels compared with the full-length channels. These results provide a direct demonstration that the N terminus of aKv1.1 contributes significantly to the stability of the full-length tetrameric channel.

Tryptic Release of Tetrameric T1 Domain from the Full-length Channel—We next addressed the question of how the T1 domain is integrated into the intact channel by partial proteolytic digestion of the full-length channel. Previous experiments on the T1 domain expressed in bacteria showed that the T1 domain tetrameric core is resistant to trypsin proteolysis (1). To assess whether the T1 domain remains resistant to trypsin proteolysis within the channel and whether the linkages between the T1 domain and S1 are trypsin-sensitive, we digested membranes containing EGFP-tagged aKv1.1 channels for varying lengths of time in trypsin and probed Western blots with an anti-T1 antisera. The results (Fig. 5) show a rapid proteolysis of the channel into a T1 containing fragment of ~30 kDa, a size consistent with digestion from within the extracellular linker between S1 and S2. Eventually, the digestion further reduces the size of the T1-containing fragments to ~12 kDa, which is equivalent to the size of the trypsin-resistant core of the aKv1.1 cytoplasmic N terminus after overexpression in bacteria. These results suggest that within the channel there is a folded and assembled core of T1 domain that is resistant to tryptic digestion, similar to the tetrameric T1 domain expressed as a soluble protein. Furthermore, the linkages between the T1 domain and S1 are proteolytically sensitive, although considerably less so than the region between S1 and S2.

Given that the channel polypeptide backbone can be cleaved between the T1 domain and S1, we sought to determine whether this cleavage resulted in the release of the T1 domain as a soluble tetrameric protein or whether other elements continue to retain the domain on the channel. After the tryptic digestion of membranes containing Kv channels, we spun down the membranes to isolate a soluble fraction. We then separated the soluble fraction by SEC-FPLC (Fig. 5*B*). Western blots of the FPLC fractions reveal that the 12-kDa peptide from T1 is present in the tetrameric fraction. The tetrameric, trypsinized T1 fractions were pooled and probed ith a panel of cross-linkers and compared with a T1 construct expressed in bacteria. Similar to the bacterially expressed T1 tetramer, the domain released from the intact channel is selectively cross-linked to a tetramer with disuccinimidyl glutarate(Fig. 5*C*). These results demonstrate that proteolytic cleavage of the T1 to S1 linker is sufficient to release the T1 domain from the full-length channel as a tetramer that is structurally similar to the clonal T1 tetramer expressed in bacteria and used for structural studies.

DISCUSSION

A unique feature of the voltage-gated potassium channel family is the presence of the highly conserved T1 domain at the cytoplasmic N terminus. This domain is common to all Kv subfamilies. The coupling of this domain to a six-transmembrane channel likely provides a molecular advantage to these channels that is related to the evolutionary success and abundance of this gene family. To understand the fundamental advantages of having a T1 domain on the cytoplasmic N terminus of Kv channels, we seek to understand the nature and consequences of molecular interactions within this domain and between this domain and other regulatory elements. In this article we have focused on the importance of T1 tetramerization for channel assembly and the integration of the T1 domain into the full-length channel.

By using a genetic screening strategy with yeast two-hybrid T1 fusion proteins, we have identified a large number of T1 domain mutations that affect T1-T1 interactions. The identified mutations alter the sequence of highly conserved residues within the T1 domain, clearly highlighting the importance of these residues for T1 function. Because the yeast two-hybrid screen is a genetic screen of randomly generated mutants, the results represent independent confirmation of the importance of the conserved amino acids for the structural integrity of the T1 subunit fold and the subunit interfaces for tetramer assembly. Integration of the mutations into full-length subunits re-

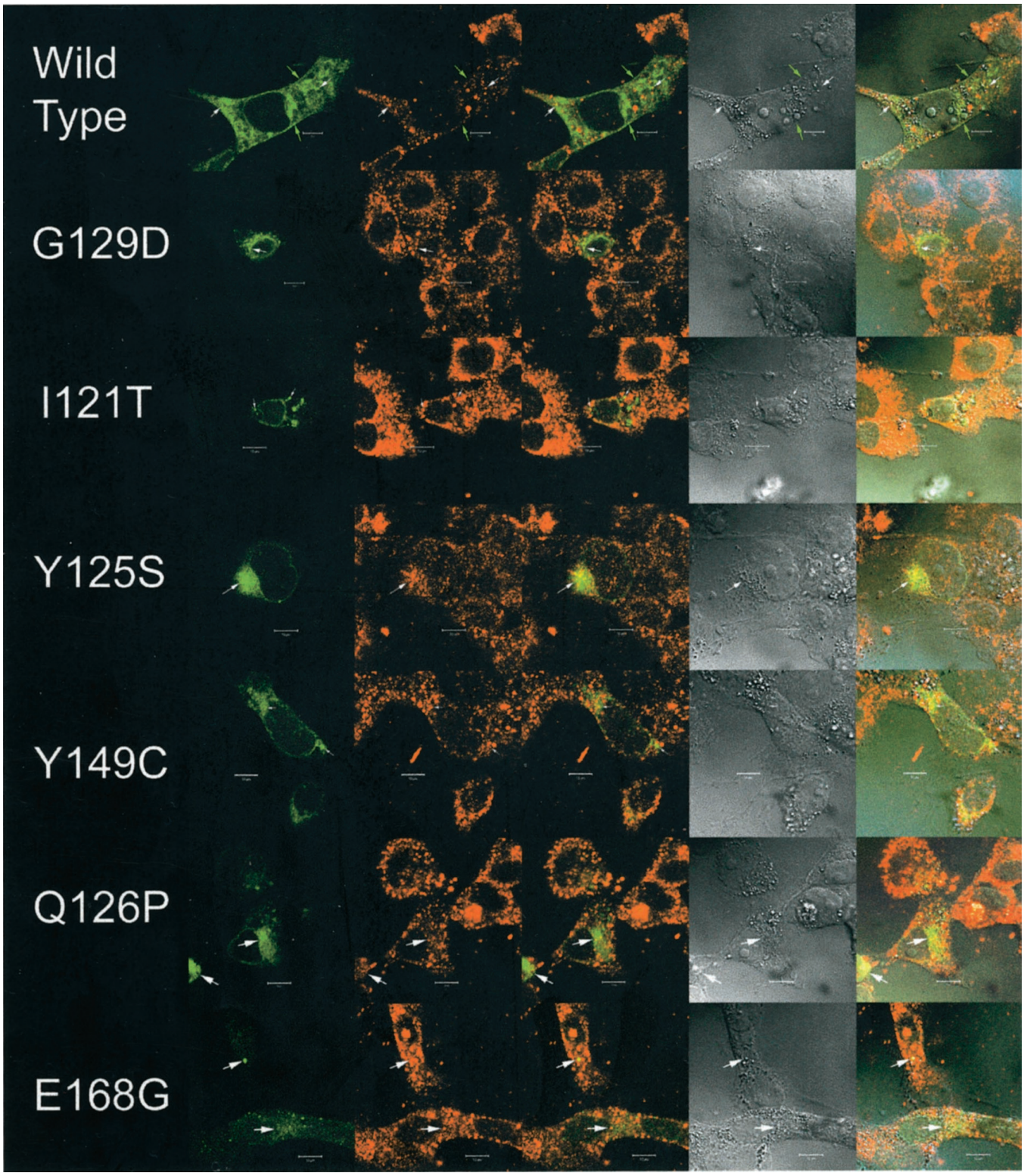

FIG. 3. **Confocal microscopy images of subcellular targeting of T1 mutations in full-length channel C-terminally tagged with EGFP expressed in COS7 cells.** In these experiments, COS7 cells were used for their morphology. The cell is large, which allows for easy visualization of the cell surface and interior space. Membrane targeting of the mutant channel is apparent; however, the fluorescence is proportionally higher in perinuclear compartments than elsewhere in the cell. In other mutations, there is a more dramatic loss of EGFP fluorescence, but the basic pattern remains the same. These micrographs are taken from mid-cell sections to more clearly demonstrate the enhanced perinuclear staining of T1 mutant channels. Immunocytochemistry to the endoplasmic reticulum marker protein, calnexin, is used to identify the endoplasmic reticulum in a representative colabeled cell (39, 40). The lane order for each Kv1.1 channel transfection (from top to bottom) is: 1, GFP channel fluorescence; 2, calnexin antisera labeled with a secondary antibody, rhodamine conjugate; 3, double channel recording (of lanes 1 + 2); 4, transmission photography; and 5, triple channel recording (of 1 + 2 + 4).

sults in a dramatic loss of functional channel expression both for amino acids that are needed for the subunit fold and amino acids that participate in subunit association to make quaternary structure. It then seems that the monomeric subunit folds independently and that the folded T1 facilitates the subunit interactions that yield tetrameric channel. T1 acts as a subunit

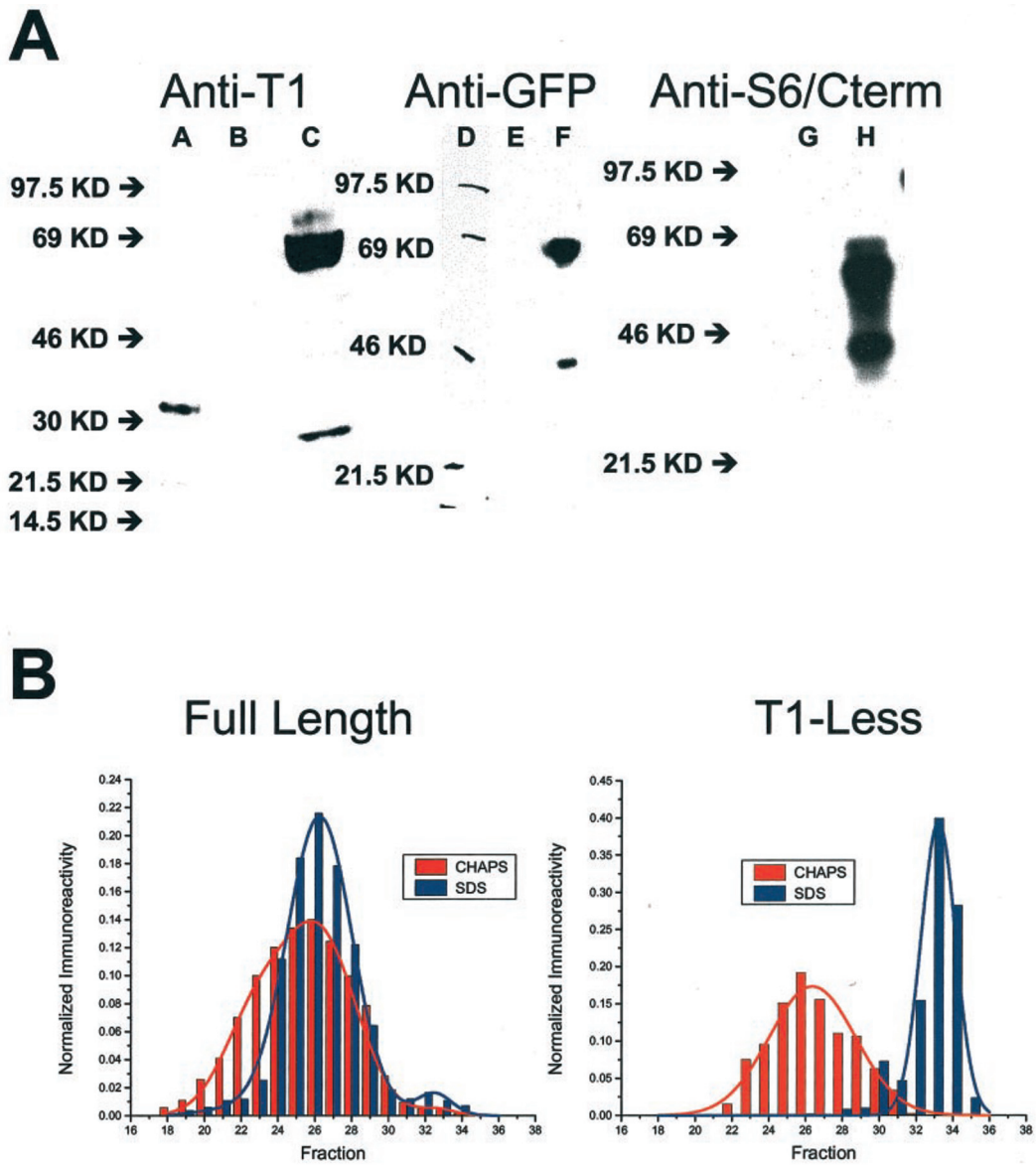

FIG. 4. **Channels lacking the N terminus of aKv1.1 are destabilized to denaturants relative to full-length channel.** *A*, Western blots of stable cells transfected with aKv1.1 show reactivity to both N- and C-terminal epitopes. Membrane preparations of transfected and untransfected cells were solubilized with 1% SDS, separated by SDS-PAGE (10%), and transferred to membranes for Western blot detection with either antisera generated to a bacterially expressed aKv1.1 T1 domain (N-terminal epitope, *Anti-T1*), a commercially available GFP antisera (C-terminal epitope, *Anti-GFP*), or a peptide epitope from the C terminus corresponding to amino acids 429–464 (C-terminal epitope, *Anti-S6/Cterm*). No antisera shows recognition toward a lane of untransfected CHO cell membranes (see *lanes B*, *E*, and *G*), whereas all antisera recognize the full-length channel subunit, migrating at 63–66 kDa (see *lanes C*, *F*, and *H*). Each of the lanes for transfected cells contains a minor band of truncated channel, and each minor band shows a distinct molecular mass (see *lanes C*, *F*, and *H*) and immunoreactivity, because *lane C* is an epitope map for the N terminus and *lanes F* and *H* are epitope maps for the C terminus. This suggests that the full-length channel subunit is cleaved by a protease of unknown specificity or cellular location to yield a minimum of two polypeptides that retain either the N-terminal epitope from T1 (as in *lane C*) or the C-terminal epitope (as in *lanes F* and *H*). The resultant channel fragments migrate at relative molecular masses of 28 kDa for the N-terminal piece and 44 kDa for the C-terminal piece, and this latter molecular mass correlates well with the smaller fragment observed from the FPLC analysis of aKv1.1 in Fig. 2, in which the GFP-positive fragment there migrates at ~45 kDa. Similarly, the 28 kDa of the N-terminal fragment is reactive with antisera to the T1 domain. This mass is sufficient to include the entire N-terminal cytoplasmic domain and the S1 domain thereby also explaining its retention in the membrane fraction. We cannot declare the exact N or C terminus of either fragment without further characterization, but the sum of these molecular masses (72 kDa) may well represent all of the channel subunit, because it migrates at 66 kDa as an uncleaved polypeptide. Thus, we present here sufficient evidence to show that these fragments represent two portions of the channel subunit with the following characteristics: the fragment that is reactive with the antisera to T1 contains the majority of the N-terminal domain and part or all of S1, and the fragment that is reactive with the GFP antisera is of sufficient molecular mass that it harbors most if not all of the C-terminal domain and the transmembrane segments, S2–S6. *KD*, kilodaltons. *B*, membrane preparations of stable cells expressing aKv1.1-EGFP were solubilized with 1% CHAPS either with or without 1% SDS. Solubilized channel proteins were fractionated by SEC-FPLC, and the locations of the subunits either with or without the channel N terminus were determined by Western blotting with anti-GFP antisera. As shown in Figs. 2 and 4*A*, the channel proteins lacking T1 immunoreactivity migrate at a lower molecular mass than the full-length channels. Histograms showing the amount of either protein form in each fraction are plotted. In CHAPS buffer, both forms of the channel are found in tetrameric fractions; however, the addition of SDS selectively disrupts the T1-less channels to the monomer size.

tether by providing spatial orientation and sufficient binding interaction to greatly enhance the level of tetrameric channel, and therefore channel on the cellular surface, both as shown here and in the experiments using an artificial tetrameric scaffold (31).

It is important to note that there are inherent differences in

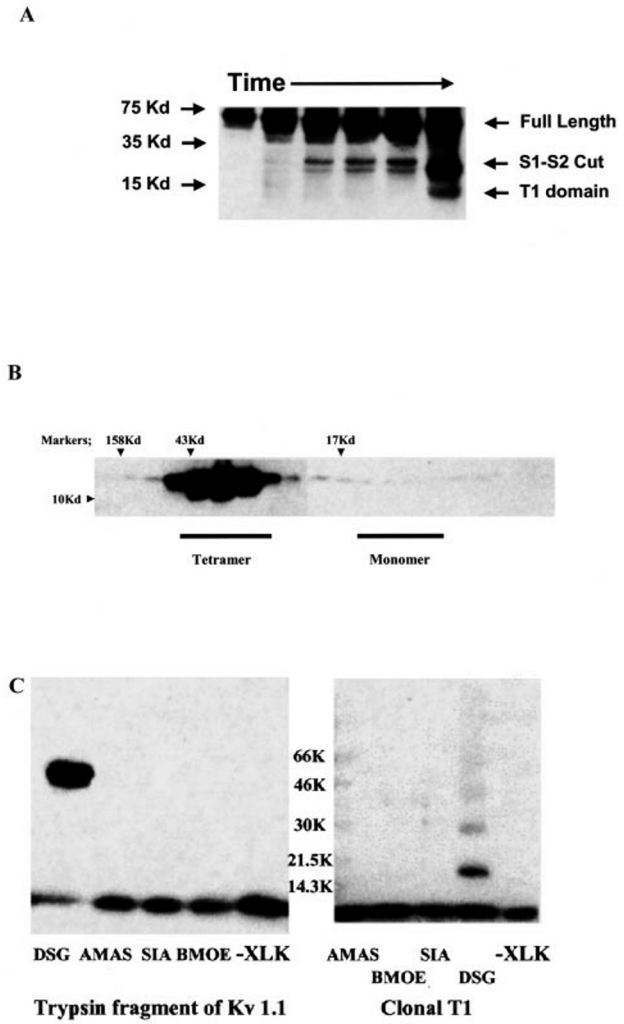

FIG. 5. **Release of tetrameric T1-containing peptides from intact and membrane-integrated Kv1.1.** *A*, time course of trypsin digestion (Kv1.1). Membrane preparations of Kv1.1 were digested with trypsin at 23 °C for 0, 2, 4, 8, 12, and 16 h, separated by SDS-PAGE (10%), and Western blotted with anti-T1 antisera (*lanes 1–6*); the same amount of digested protein was loaded into each lane. The appearance of a trypsin-resistant T1 core fragment is indicated by the *arrow*. Intermediate bands of 28.6 and 31.4 kDa begin to appear at 4 h and persist and increase in amount up to 16 h, and a smaller molecular mass band of 22 kDa or less appears by 16 h. The parent band does not decrease in intensity as might be expected, but instead it concomitantly broadens to represent a much greater range of molecular masses from ~47–56 kDa at 2 h to the widened range of ~35–62 kDa by 12 h. We believe that the reason for the intensity that remains the same and the broadening of the parent band is the aggregation of trypsinized cleavage products. The intermediate-sized fragments represent an N-terminal fragment from aKv1.1 that likely includes the S1 transmembrane segment as well with the expected trypsin cleavage positions within the S1-S2 loop. *Kd*, kilodaltons. *B*, the tryptically released T1 core is a soluble tetramer. Membrane preparations of Kv1.1 were trypsinized, soluble released peptides were separated on an SEC-FPLC column, and the locations of anti-T1 immunoreactive material were determined by Western blotting of 15% SDS-PAGE gels. The trypsin-resistant T1 core elutes from the SEC column in fractions expected for the tetrameric form of the T1 domain. Note that the protein is barely detectable in the monomer fractions, showing that the T1 domain is released from the intact channel as a tetramer. *C*, the pooled T1 tetramer fractions from the SEC separation after trypsin release from the channel were cross-linked with a panel of cross-linkers and compared with bacterially expressed T1 domain protein. These cross-linkers bridge two amino acid side chains covalently as follows: Cys to Cys, (bismaleimidoethane (*BMOE*)), Cys to Amino (*N*-α-[maleimidoacetoxy]succinimide ester (*AMAS*), *N*-succinimidyl iodoacetate (*SIA*)), or Amino to Amino (disuccinimidyl glutarate (*DSG*)) at <8-Å proximity. Bismaleimidoethane, *N*-α-[maleimidoacetoxy]succinimide ester, and *N*-succinimidyl iodoacetate are unable to cross-link either protein. Disuccinimidyl glutarate is capable of cross-linking both the released T1 core and the bacterially expressed T1 domain to a tetramer, suggesting that the T1 protein released from the channel has a similar structure to the bacterially expressed protein. Here, in identical gels aligned by molecular mass markers we can compare directly the trypsinized fragment and clonal T1 expressed in bacteria. Although these fragments are quite similar in size, it does appear that the trypsin-released fragment is slightly larger. In the lanes in which the respective T1 domains are cross-linked with disuccinimidyl glutarate, there is 100% tetramer for the trypsin-released fragment and a mix of dimer, trimer, and tetramer for the clonal T1. The two possible reasons for this are that the ratios of cross-linker/protein were different in these two experiments, with the ratio for the trypsin fragment much higher, or it is possible that the extra molecular mass in the trypsin fragment provides increased stability and decreased dynamics for the folded structure that permits cross-linking to take place.

the assays used to identify mutants and to evaluate the effects of these mutations on channel assembly. The yeast two-hybrid experiments screen for the presence or absence of heteromultimer assembly between point mutant and wild-type subunits in a relatively qualitative way. In contrast, the expression of channels in oocytes, CHO, or COS cells for electrophysiology, biochemistry, and fluorescence microscopy examines the ability of mutant subunits to form homotetrameric channels. Despite these differences, we were struck by the similarity in magnitude of the mutational effects in the yeast screen and the oocyte electrophysiology. We propose the following explanation based on mechanistic arguments. The yeast two-hybrid screen is an *in vivo* competition assay in which the Gal4 fusion construct with the mutant T1 subunit must compete with Gal4 wild-type subunit fusion to form a heteromeric and active transcription factor against the highly favored tendency of the wild-type fusion to self-associate to an inactive transcription factor. In contrast, in CHO expression studies the mutant subunits are not in competition with other subunits; however, there does seem to be a competition between tetramer formation and degradation of the monomeric subunit.

Thus, normal channel assembly may be a highly optimized cellular process in which a balance between tetramerization and monomer degradation has physiological significance in the level of channel gene expression ultimately realized at the cell surface. The resultant combination of decreased tetramerization and increased proteolysis of T1 mutant subunits results in a dramatic decrease in the amount of tetrameric channel protein, as shown by electrophysiology, fluorescence microscopy, and FPLC. Our results therefore strongly support the importance of T1 domain tetramer formation in promoting assembly and transport to the cell surface of functional Kv channels against a competitive process that involves the proteolytic scavenging of monomeric subunits.

In addition, we have identified a small fraction of the Kv subunit protein in our overexpression system that lacks the N-terminal epitope recognized by our antibody. Given that the subunit protein without T1 reactivity was found initially in the tetrameric fraction of a membrane preparation, we suspect that the cleavage is occurring after channel tetramerization. Hence, we believe that these are channels in which individual subunits have been proteolytically cleaved in or near the T1-S1 linker region. Here, we have not addressed the mechanism for the generation of the channels lacking T1, but instead we have used this as a tool to examine the structural stability of channels that were folded by a completely normal mechanism but still lack the cytoplasmic N-terminal domain. We suggest that the cleavage may have biological relevance, because no exogenous protease has been added, and we have taken reasonable precautions to prevent the activity of cellular proteases during harvesting and membrane solubilization. An interesting question is whether this specific cleavage is a fundamentally different process from the mechanism responsible for the increased

proteolytic loss of T1 mutant channel monomers, which can be observed in the mutants displayed in Fig. 2. Our future studies will be testing these ideas and further examining the extent to which T1 post-translational processing is a normal part of Kv channel biology *in vivo*.

Regardless of the normal role for the proteolytic removal of the T1 domain from Kv channel subunits, it is clear that channels lacking the N-terminal sequence are structurally less stable than channels with the domain. These results suggest that the T1 domain is contributing significantly to the stability of the full-length channel. By using tryptic digestion, we are able to demonstrate that the T1 domain exists within the core of the channel as a stably assembled tetramer. The continued interaction of subunits within the core of the T1 interface likely provides a partial explanation for the increased stability of T1-containing channels.

We can release intact tetrameric T1 from an intact channel with trypsin digestion alone, albeit over a period of several (16) hours at room temperature. The soluble protein that is shown in Fig. 5*B* is derived from cellular membranes that were trypsinized and then centrifuged prior to loading the soluble fraction, without CHAPS solubilization, onto a gel filtration column. These bands clearly represent freely soluble and released tetramer with all four T1-S1 linkages cleaved by trypsin. We have not performed the FPLC analysis on the pelleted membranes, because it is more difficult to examine the membrane fraction for associated but *fully cleaved* tetrameric T1. This experiment will result in a mix of tetramers, because it will retain a fraction of T1 that is partially cleaved with only one, two, or three of the subunits cleaved, as well as any fully cleaved tetramer that remains bound by noncovalent forces. All of these species will elute from the column with the full-length tetrameric channel, but we cannot distinguish them from one another by subsequent SDS-PAGE because each of these species contains the same clipped monomeric fragment of ~10 kDa on the gel.

Therefore we are left only with the fact that we can release a T1-like tetramer without the use of any denaturants, chaotropes, or dissociating agents such as temperature or pH. This finding suggests that the T1 domain is only loosely associated with any other part of the channel under conditions in which there is no membrane potential, and it dissociates from the membrane-integrated segments once its polypeptide linkage to S1 is cleaved.

Our studies also provide preliminary information about the nature of the linkages between the T1 domain and the transmembrane region of the channel. Tryptic digestion studies reveal that the region linking the T1 domain to the first transmembrane domain, S1, is accessible to cleavage by trypsin. The rate of this cleavage is clearly slower than the tryptic cleavage of the S1-S2 loop region, a poorly conserved region of the channel, but faster than the digestion of the core of the T1 domain itself, which is trypsin-resistant. It is unclear whether cleavage of the linker between S1 and S2 is a prerequisite for tryptic cleavage of the linker between T1 and S1, because it always occurs prior to the cleavage to generate T1. The relatively slow cleavage of the T1-S1 linker in addition to the relatively high level of sequence conservation in the T1-S1 linking region suggest that this region is unlikely to be completely accessible and unstructured in the channel.

Furthermore, the trypsin-cleaved and -released polypeptide seems to be of a single molecular mass by SDS-PAGE, both of the digest itself and the FPLC-purified tetramer (Fig. 5), suggesting that there are preferred sites among the four potential trypsin cleavage sites to the N-terminal side and the six lysine or arginine residues to the C-terminal side of the T1 residues within the intact channel polypeptide. Therefore, we conclude that only a small section of polypeptide is accessible and that the linker is partly buried and structured, or dynamic in structure. A structured T1-S1 linker could provide a mechanism for linking conformational changes in the T1 domain to gating changes within the transmembrane pore region of the channel. Future studies will be needed to determine the structural nature of the T1-S1 linker and whether conformational changes in the T1 domain are linked to the transmembrane gating regions through this linker or more directly through interactions between the T1 domain and the channel gating machinery.

Based on these and other studies, we propose that the molecular advantages of coupling a T1 domain to a six-transmembrane channel include: 1) tetramerization of the T1 domain drives channel assembly through enhanced formation of tetrameric intact channels, 2) T1 tetramerization is a selective process that can restrict the number of possible channels made in a cell based on the specificities of the T1 assembly interfaces, 3) the T1 domain adds stability to the formed ion channel through interactions within the domain and possibly through interactions with other channel elements, 4) small conformational changes in the T1 domain structure are capable of regulating channel gating properties, and 5) the T1 domain provides a linkage site for the interactions of modulatory auxiliary proteins with the channel gates through transdomain conformational signaling. These molecular advantages likely provide part of the framework for understanding the function and evolutionary success of the Kv channel family.

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