[H. Ueyama, H. Hamada, N. Battula, T. Kakunaga, *Mol. Cell. Biol.* **4**, 1073 (1984)].
28. The multidrug-resistant J2c cell line is a derivative of the drug-sensitive S3 HeLa cell line obtained by the selection of mutagenized S3 cells in doxorubicin. The J2c cell line is about fivefold more resistant to doxorubicin relative to S3 and does not overexpress P-glycoprotein (R. Baker, personal communication).
29. J. Devereux *et al.*, *Nucleic Acids Res.* **12**, 387 (1984).
30. D.-F. Feng and R. F. Doolittle, *J. Mol. Evol.* **25**, 351 (1987). The final alignment is produced by a series of progressive, pairwise alignments, beginning with the two most similar sequences and continuing until all sequences have been included in the final alignment. Before alignment, the sequences are first clustered by similarity to produce a dendrogram that directs the order of the subsequent pairwise alignments. Although this dendrogram is not a phylogenetic tree, the horizontal branch lengths are inversely proportional to the degree of similarity between the sequences. Based on the FASTA search, the following protein sequences (with their GenBank/EMBL accession numbers) were selected for alignment with MRP (Hum/MRP): cystic fibrosis transmembrane conductance regulators from humans (Hum/CFTR, A30300), cow (Bov/CFTR, M76128), mouse (Mus/CFTR, M69298), and dogfish (Squ/CFTR, M83785 and M76974); P-glycoproteins from *Leishmania* (Lei/PgpA, A34207), humans (Hum/Mdr1, A25059 and Hum/Mdr3, JS0051), Chinese hamster (Cru/Pgp1, M60040; Cru/Pgp2, M60041; and Cru/Pgp3, M60042), *Drosophila* (Dro/Mdr49, M59076 and Dro/Mdr65, M59077), *Caenorhabditis elegans* (Cel/PgpA, X65054 and Cel/PgpC, X65055), *Entamoeba histolytica* (Enh/Pgp1, M88599 and Enh/Pgp2, M88598), *Arabidopsis thaliana* (Ath/Pgp1, X61370), and *Plasmodium falciparum* (Pfa/Mdr1, A32547); MHC class II–linked peptide transporters from human (Hum/Tap2, M74447) and mouse (Mus/Tap1, M55637); the mating factor exporter from yeast (Ysc/Ste6, S05789, S14174, and S05872); the bacterial toxin exporters from *Escherichia coli* (Eco/HlyB, M10133 and M12863) and *Pasteurella haemolytica* (Pas/LktB, M20730); and the heterocyst differentiation protein from *Anabaena* sp. (Ana/HetA, M31722).
31. The inserts of four λgt11 cDNA clones with a minimum of 400 nucleotides of overlapping sequence were subcloned into pGEM-3Zf(+) (Promega), and the nucleotide sequences of both strands were determined by the dideoxynucleotide chain termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. We confirmed the continuity of the total sequence compiled from the four clones (5009 nucleotides) by using the reverse polymerase chain reaction to amplify overlapping segments of MRP mRNA that spanned the entire sequenced region. The compiled sequence contained a single open reading frame encoding a protein of 1522 amino acids. The putative translational initiation codon of this open reading frame was preceded by an in-frame termination codon located 141 nucleotides upstream (5′ to the coding sequence). The length of the 5′ untranslated region of the mRNA was predicted by partial sequencing of seven additional cDNA clones that were independently isolated and complementary to the 5′-proximal portion of MRP mRNA. Three clones contained untranslated leader sequences of 204 nucleotides. The other four contained leader sequences of 222 or 225 nucleotides. The location of the 5′ end of the mRNA was also determined by ribonuclease H mapping, which indicated that the untranslated leader sequence was 200 to 225 nucleotides long. The MRP cDNA sequence will appear in sequence databases under accession number LO5628.
32. P. Klein, M. Kanehisa, C. DeLisi, *Biochim. Biophys. Acta* **815**, 468 (1985).
33. M. E. Harper and G. F. Saunders, *Chromosoma* **83**, 431 (1981). Metaphase chromosomes on slides were denatured for 2 min at 70°C in 70% deionized formamide and 2× SSC and then dehydrated with ethanol. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulfate, 2× SSC (pH 6), sonicated salmon sperm DNA (20 μg/ml), and ^{3}H-labeled MRP cDNA (0.2 μg/ml). The cDNA probe was uniformly labeled to a specific activity of 8.5×10^8 cpm per microgram of DNA with [^{3}H]thymidylate deoxynucleotide and [^{3}H]adenylate deoxynucleotide (Du Pont Biotechnology Systems) and denatured in the hybridization solution at 70°C for 5 min. Probe solution (50 μl) was placed on each slide and incubated at 37°C overnight. After hybridization, the slides were washed in 50% deionized formamide and 2× SSC, followed by 2× SSC (pH 7), and then dehydrated sequentially in ethanol. The slides were coated with emulsion (NTB/2; Kodak) and developed after exposure for 5 weeks at 4°C. Chromosomes were stained with a modified fluorescence, 0.25% Wright's stain procedure [C. C. Lin, P. N. Draper, M. Braekeleer, *Cytogenet. Cell Genet.* **39**, 269 (1985)].
34. We thank R. M. Baker (Roswell Park Memorial Institute, Buffalo, NY) for providing the HeLa cell lines, S. Mirski for discussions, and M. Vasa and K. Sparks for technical contributions. Supported by grants from the Medical Research Council (MRC) of Canada (to S.P.C.C. and R.G.D.) and the National Cancer Institute of Canada (to J.H.G.). Work in A.M.V.D.'s laboratory is supported by the Canadian Networks of Centres of Excellence Program. J.E.M. and C.E.G. are recipients of MRC postdoctoral fellowships, and E.U.K. is the recipient of an MRC studentship.

8 June 1992; accepted 9 September 1992

Expression of an Inward-Rectifying Potassium Channel by the *Arabidopsis KAT1* cDNA

Daniel P. Schachtman, Julian I. Schroeder, William J. Lucas, Julie A. Anderson, Richard F. Gaber

Inward-rectifying potassium channels located in the plasma membrane of higher plant and animal cells contribute to cellular homeostasis and excitability. The genes encoding this specific class of K^+ channels have not been functionally identified. This report shows that a single messenger RNA transcript from the *Arabidopsis thaliana KAT1* complementary DNA confers the functional expression of a hyperpolarization-activated K^+ channel in *Xenopus* oocytes. The channels encoded by *KAT1* are highly selective for K^+ over other monovalent cations, are blocked by tetraethylammonium and barium, and have a single-channel conductance of 28 ± 7 picosiemens with 118 millimolar K^+ in the bathing solution. These functional characteristics, typical of inward-rectifying K^+ channels in eukaryotic cells, demonstrate that *KAT1* encodes an inward-rectifying K^+ channel.

Analysis and manipulation of cDNAs encoding outward-rectifying K^+ channels (*1*) has led to an understanding of how components of the primary protein structure contribute to functional characteristics such as voltage-dependent activation (*2*) and ionic conductivity (*3*). Although inward-rectifying K^+ channels regulate excitability in animal cells (*4*) and K^+ uptake in higher plant cells (*5*, *6*), little is known about the protein structure of this class of ion channels.

Two cDNAs, *AKT1* and *KAT1*, were cloned from the higher plant *Arabidopsis thaliana* (*7*, *8*) by the complementation of *Saccharomyces cerevisiae* mutants deficient in K^+ uptake (*9*). AKT1 and KAT1 share some amino acid similarity to outward-rectifying K^+ channels in a voltage-sensing domain (S4), in an ion-conducting pore-forming region (H5), and in the predicted topology of the core region of the protein (*7*, *8*). Despite structural similarity between AKT1 and KAT1 and outward-rectifying K^+ channels, these plant genes completely restored K^+ uptake to yeast mutants. From patch clamp studies on guard cells, K^+ uptake into plant cells has been ascribed to proton pump–driven K^+ influx through inward-rectifying K^+ channels (*5*, *10*–*12*). Therefore, characterization of the *KAT1* cDNA by heterologous expression in *Xenopus laevis* oocytes was initiated to determine whether the protein encoded by *KAT1* functions as a voltage-activated inward-rectifying K^+ channel.

Uninjected or water-injected control oocytes were analyzed in all experiments and showed only small currents in response to hyperpolarizing pulses (Fig. 1, A and D) that activated at membrane potentials more negative than −145 ± 14 mV [n = 20; mean ± SD]. These endogenous currents have been suggested to be carried by chloride ions (*13*). In 65 oocytes (from ten frogs) injected with mRNA synthesized from the *KAT1* cDNA (*14*), large inward currents were measured that were activated by hyperpolarization of the membrane potential to values more negative than −102 ± 13 mV (n = 14) (Fig. 1, B and D). Currents were not elicited by depolarization of the membrane potential in injected oocytes (Fig. 1, C and D). Inward current magnitude was 1.2 ± 0.5 μA (n = 23) at

D. P. Schachtman and J. I. Schroeder, Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093.
W. J. Lucas, Department of Botany, University of California, Davis, Davis CA 95616.
J. A. Anderson and R. F. Gaber, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208.

−150 mV, 18 to 40 hours after injection with mRNA. The time- and voltage-dependent activation of the current in oocytes injected with *KAT1* mRNA is characteristic of inward-rectifying K^+ channel currents in higher plants (*5, 10–12*).

We determined the ion selectivity of the *KAT1*-induced hyperpolarization-activated currents by measuring the change in amplitude of the current in whole oocytes when 115 mM KCl in the bathing solution was replaced with equimolar concentrations of RbCl, NH_4Cl, CsCl, NaCl, or LiCl (*15*). Replacement of KCl with CsCl, LiCl, and NaCl resulted in a large reduction of the hyperpolarization-activated current (Fig. 2, B and D and Table 1). However, Rb^+ and NH_4^+ showed a relative conductance of approximately 30% with respect to the K^+ conductance (Table 1 and Fig. 2, C and D). After re-exposure of oocytes to K^+ Ringer solution, 85 ± 6% of current was recovered after removal of NH_4^+, and 95 ± 23% of current was recovered after removal of Cs^+. Within the limits imposed by the two-electrode voltage clamp method (*16*), reversal potentials of tail currents were determined to be −6 ± 6 mV (n = 8) in 118 mM K^+ and −48 ± 4 mV (n = 3) in 12 mM K^+ (*15*). The change in external K^+ concentration from 118 to 12 mM would result in a reversal potential shift of −52 mV for a perfectly selective K^+ channel, after correction for ionic activities (*10*). A reversal potential of −30 ± 8 mV (n = 4) was measured with 115 mM NH_4^+ in the bath, giving rise to a permeability ratio of $P_{NH_4^+}/P_{K^+}$ = 0.42. These data show that inward-rectifying *KAT1*-associated currents are selective for K^+ over other monovalent cations. The ionic selectivity of the inward-rectifying current (Table 1) is similar to K^+ selectivities of inward-rectifying K^+ channels in various organisms (*4, 5, 10–12*).

The ability of *KAT1* to restore growth to *S. cerevisiae* mutants on K^+-limiting medium is inhibited by the K^+ channel blockers tetraethylammonium (TEA^+) and barium (*8*). When the extracellular bath solution was perfused with 115 mM KCl containing TEA^+ or Ba^{2+}, *KAT1*-evoked currents in oocytes were reduced (Fig. 3). At a membrane potential of −130 mV, 10 mM TEA^+ blocked 81 ± 10% (n = 4), 1 mM Ba^{2+} blocked 38 ± 18% (n = 2), and 10 mM Ba^{2+} blocked 70 ± 11% (n = 4) of the inward-rectifying K^+ current. After perfusion with K^+ Ringer solution that did not contain TEA^+, 90 ± 11% of the initial current was recovered, whereas 68 ± 25% of the initial current was recovered after the removal of 10 mM Ba^{2+}. The blockage of the *KAT1*-mediated current by Ba^{2+} and TEA^+ is similar to that observed for inward-rectifying K^+ channels in plant and animal cells (*5, 17*).

Table 1. Conductance ratios of the KAT1 channel to monovalent cations. Values (±SD) were measured at the end of 1.5-s pulses at a membrane potential of −130 mV and are relative to the K^+ conductance.

	K^+	Rb^+	Na^+	Cs^+	Li^+	NH_4^+
% K^+ conductance	100	28 ± 13	7 ± 8	9 ± 11	6 ± 3	30 ± 12
Number of oocytes	25	5	4	4	3	11

To analyze single-channel properties of *KAT1*-induced K^+ currents, we studied oocytes expressing *KAT1* cDNA and uninjected control oocytes by cell-attached patch clamp recordings (*18*). Typical *KAT1*-induced single-channel currents elicited by membrane hyperpolarization are shown in Fig. 4. These ion-channel currents were not detected in 17 cell-attached patches from uninjected oocytes. The slope conductance of the KAT1 inward-rectifying K^+ channel was 28 ± 7 pS with 118 mM K^+ on the extracellular membrane side. The single-channel conductance measured in these experiments is in the range of inward-rectifying K^+ channel conductances in guard cells and barley aleurone cells (*5, 12*).

Other properties of *KAT1*-mediated inward-rectifying K^+ channels were also characteristic of inward-rectifying K^+ channels described in higher plant cells. *KAT1*-induced currents showed no significant inactivation during continuous hyperpolarizations of 2 min (*10*), and the activation potential of the channels depended only moderately on the extracellular K^+ concentration (*19*), as in guard cells (*10, 20*).

The voltage- and time-dependent activation, ionic selectivity, blockage by TEA^+ and Ba^{2+}, single-channel conductance, and lack of inactivation of the KAT1 current show that this cDNA encodes an inward-rectifying K^+ channel similar to those found in higher plants (*5, 10–12*).

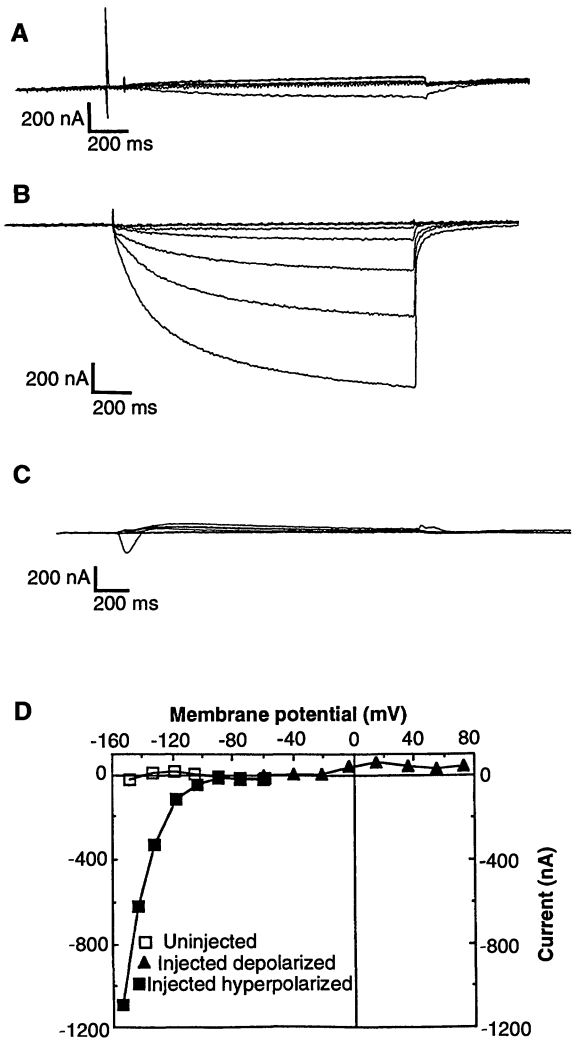

Fig. 1. *Arabidopsis thaliana KAT1* cDNA confers functional expression of hyperpolarization-activated currents in *Xenopus* oocytes. Currents were elicited in response to hyperpolarizing pulses from a holding potential of −60 mV in uninjected oocytes (**A**) and in oocytes injected with *KAT1* mRNA (**B**) with K^+ Ringer solution in the bathing medium (*15*). (**C**) Currents elicited in response to depolarizing pulses from a holding potential of −60 mV in oocytes injected with *KAT1* mRNA. (**D**) Currents at the end of pulses shown in (A), (B), and (C) are plotted as a function of applied voltage-pulse potentials.

Whether inward-rectifying K^+ channels in animal cells are related in structure to the KAT1 channel remains to be determined. The extensive amino acid sequence identity between *AKT1* (*7*) and *KAT1* (*8*) suggests that there may be a family of genes encoding inward-rectifying K^+ channels in the *Arabidopsis thaliana* genome. Injection of plant cRNA pools shows that oocytes also functionally express plant outward-rectifying K^+ channels in addition to the KAT1 inward-rectifying K^+ channel (*21*).

Potassium is an essential macronutrient for plant growth (*22*). Biophysical, pharmacological, and cell biological studies on guard cells have led to the suggestion that inward-rectifying K^+ channels provide the major low-affinity pathway for K^+ uptake into higher plant cells (*5*, *10*–*12*, *20*). The electrogenic proton-extruding adenosine triphosphatase (ATPase) in the plasma membrane of plant cells hyperpolarizes the membrane to sufficiently negative potentials (*23*) to open inward-rectifying K^+ channels and drive physiological fluxes of K^+ through these channels (*5*, *20*). Because inward-rectifying K^+ channels in plants show no significant inactivation (*10*), this mechanism would allow long-term proton pump–driven K^+ uptake into plant cells.

An additional property of the KAT1 channel is a significant NH_4^+ conductance (Fig. 2C). Ammonium ions are taken up by plants and used for nitrogen nutrition (*24*). Because the KAT1 channel is permeable to NH_4^+, and NH_4^+ may inhibit K^+ absorption in some cultivars (*25*), we suggest that inward-rectifying K^+ channels may also provide a pathway for ammonium influx into plant cells.

A conspicuous property of the KAT1 channel lies in its hyperpolarization-induced activation. All other cloned voltage-dependent Na^+, K^+, and Ca^{2+} channels are activated by depolarization. The conserved S4 domains in these channels (*26*) have been modeled as a central structural component that undergoes transmembrane movement in response to depolarization, thereby enabling channel opening (*2*, *27*). The KAT1 protein contains five positively charged residues located within the hydrophobic amino acids that constitute the fourth transmembrane domain (S4-like), a motif that is suggestive of the S4 domain in outward-rectifying K^+ channels (*1*, *7*, *8*, *26*). Our results indicate that an S4-like domain can also be present in a hyperpolarization-activated ion channel. There are several possibilities by which the S4-like domain in the KAT1 inward-rectifying K^+ channel may contribute to activation in response to membrane hyperpolarization. First, factors such as higher order protein structure or domains outside the S4 domain

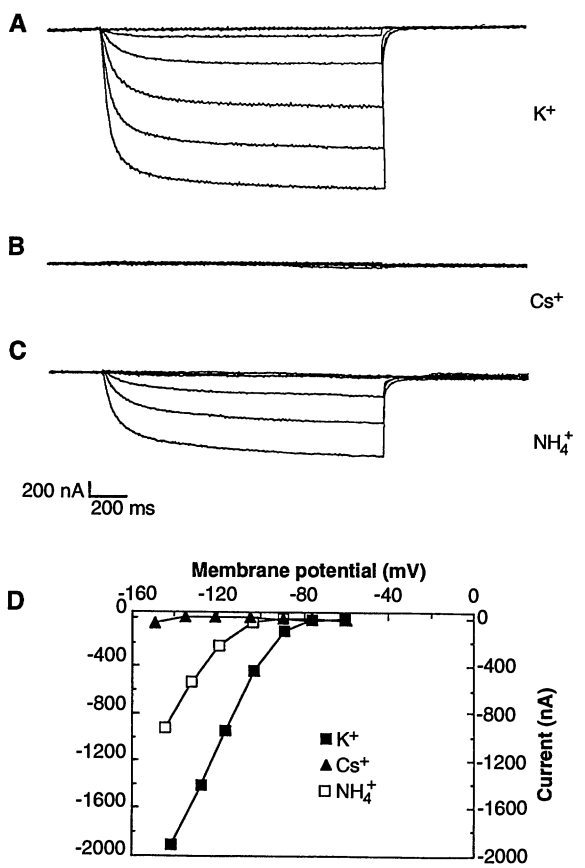

Fig. 2. Ion selectivity of inward currents in an oocyte injected with *KAT1* mRNA. Currents were evoked in response to hyperpolarizing pulses from a holding potential of −60 mV. The solution bathing oocytes contained (**A**) 115 mM KCl, (**B**) 115 mM CsCl, and (**C**) 115 mM NH_4Cl (*15*). (**D**) Currents at the end of pulses shown in (A), (B), and (C) are plotted as a function of applied voltage-pulse potentials.

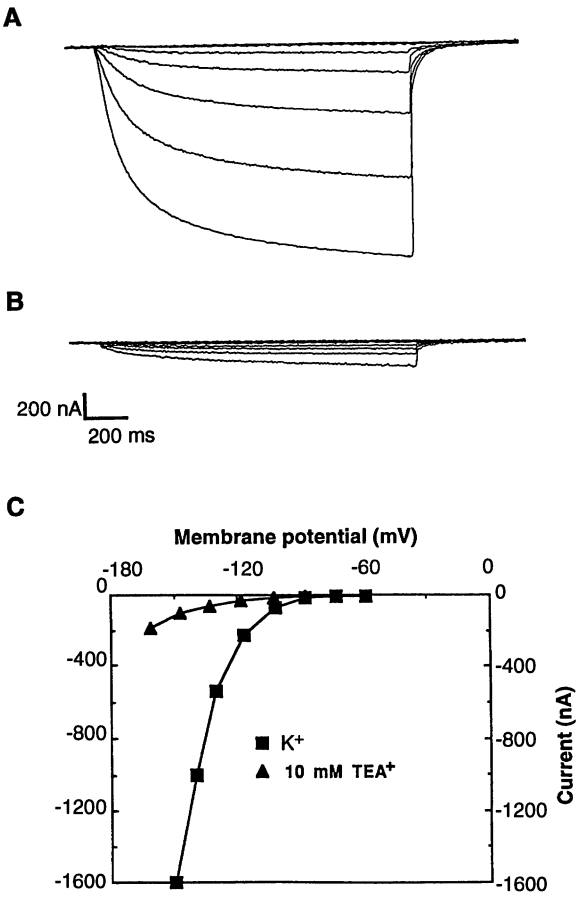

Fig. 3. TEA^+ blocks *KAT1*-mediated inward-rectifying K^+ currents. Inward currents were elicited in response to hyperpolarizing pulses with (**A**) K^+ Ringer solution bathing an oocyte and (**B**) K^+ Ringer solution supplemented with 10 mM TEA^+ in the extracellular solution of the same oocyte. (**C**) Currents at the end of pulses shown in (A) and (B) are plotted as a function of applied voltage-pulse potentials.

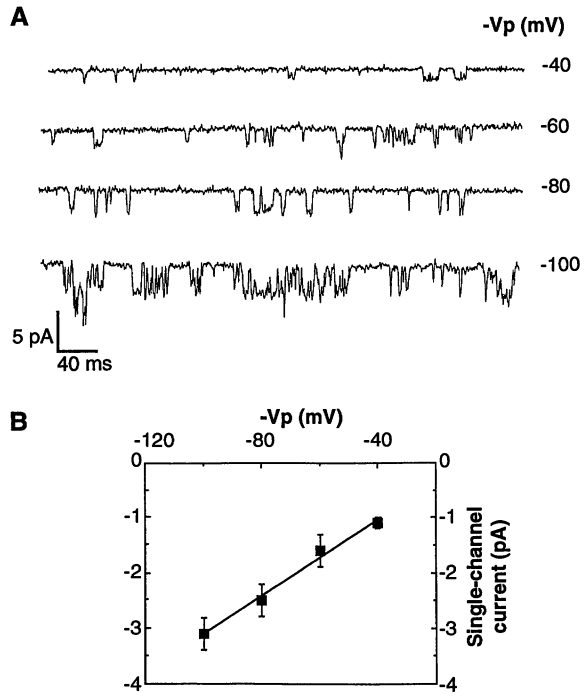

Fig. 4. Cell-attached recordings of *KAT1* mRNA–mediated single-channel currents in oocytes. (**A**) Recordings in the cell-attached configuration with K^+ Ringer solution (*15*) in the pipette, which faced the extracellular side of the membrane surface (*28*). Hyperpolarizing pipette potentials (−Vp) (*28*) are indicated to the right of recorded current traces. (**B**) Mean amplitude of single-channel currents as a function of hyperpolarizing pipette potentials (−Vp). The curve was determined by linear regression analysis, resulting in a single-channel conductance of 34 pS in the illustrated patch. Single-channel current amplitudes (mean ± SD) were determined by analysis of ≥20 openings at each potential.

may contribute to gating (*2*). Second, hyperpolarization of the membrane potential may be sufficient to initiate the movement of the KAT1 S4-like domain in the opposite direction to that suggested for depolarization-activated ion channels (*27*). Third, the S4-like domain may be inserted into the plasma membrane such that the orientation of this domain along with the other transmembrane domains in the KAT1 channel (*8*) is reversed when compared with the orientation of the membrane-spanning regions in outward-rectifying K^+ channels (*3*, *27*). Further studies will be required to test these and other possible models.

In conclusion, the *KAT1* cDNA encodes an inward-rectifying K^+ channel with properties that correlate closely to those described in higher plants. Structural similarities to outward-rectifying K^+ channels in animals suggest that these genes share common ancestral origins.

REFERENCES AND NOTES

1. D. M. Papazian, T. L. Schwarz, B. L. Tempel, Y. N. Jan, L. Y. Jan, *Science* **237**, 749 (1987); O. Pongs *et al.*, *EMBO J.* **7**, 1087 (1988); L. E. Iverson, M. A. Tanouye, H. A. Lester, N. Davidson, B. Rudy, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5723 (1988).
2. E. R. Liman, P. Hess, F. Weaver, G. Koren, *Nature* **353**, 752 (1991); D. M. Papazian, L. C. Timpe, Y. N. Jan, L. Y. Jan, *ibid.* **349**, 305 (1991); F. Bezanilla, E. Perozo, D. M. Papazian, E. Stefani, *Science* **254**, 679 (1991); N. E. Schoppa, K. McCormack, M. A. Tanouye, F. J. Sigworth, *ibid.* **255**, 1712 (1992); D. E. Logothetis, S. Movahedi, C. Satler, K. Lindpaintner, B. Nadal-Ginard, *Neuron* **8**, 531 (1992).
3. R. MacKinnon and C. Miller, *Science* **245**, 1382 (1989); R. MacKinnon and G. Yellen, *ibid.* **250**, 276 (1990); A. J. Yool and T. L. Schwarz, *Nature* **349**, 700 (1991); G. Yellen, M. E. Jurman, T. Abramson, R. MacKinnon, *Science* **251**, 939 (1991); H. A. Hartmann *et al.*, *ibid.*, p. 942.
4. B. Katz, *Arch. Sci. Physiol.* **3**, 285 (1949); S. Hagiwara and K. Takahashi, *J. Membr. Biol.* **18**, 61 (1974); S. Hagiwara, *Membrane Potential–Dependent Ion Channels in Cell Membrane, Phylogenetic and Developmental Approaches* (Raven, New York, 1983), pp. 61–86; H. Matsuda, *Annu. Rev. Physiol.* **53**, 289 (1991).
5. J. I. Schroeder, R. Hedrich, J. M. Fernandez, *Nature* **312**, 361 (1984); J. I. Schroeder, K. Raschke, E. Neher, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4108 (1987).
6. R. Hedrich and J. I. Schroeder, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 539 (1989); M. Tester, *New Phytol.* **114**, 305 (1990).
7. H. Sentenac *et al.*, *Science* **256**, 663 (1992).
8. J. A. Anderson, S. S. Huprikar, L. V. Kochian, W. J. Lucas, R. F. Gaber, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3736 (1992).
9. J. Ramos, P. Contreras, A. Rodriguez-Navarro, *Arch. Microbiol.* **143**, 88 (1985); R. F. Gaber, C. A. Styles, G. R. Fink, *Mol. Cell. Biol.* **8**, 2848 (1988); C. H. Ko and R. F. Gaber, *ibid.* **11**, 4266 (1991).
10. J. I. Schroeder, *J. Gen. Physiol.* **92**, 667 (1988).
11. J. I. Schroeder and S. Hagiwara, *Nature* **338**, 427 (1989); K. A. Ketchum, A. Shrier, R. J. Poole, *Plant Physiol.* **89**, 1184 (1989); R. Colombo and R. Cerana, *Plant Physiol.* **97**, 1130 (1991); J. Kourie and M. H. M. Goldsmith, *ibid.* **98**, 1087 (1992); K. A. Fairly-Grenot and S. M. Assmann, *Planta* **186**, 282 (1992).
12. D. S. Bush, R. Hedrich, J. I. Schroeder, R. L. Jones, *Planta* **176**, 368 (1988).
13. I. Parker and R. Miledi, *Proc. R. Soc. London* **233**, 191 (1988).
14. Oocytes were isolated as described (*21*). One day after isolation, oocytes were injected with 50 ng of *KAT1* mRNA. Recordings were made on day 1 and 2 after microinjection. Messenger RNA transcripts were synthesized in vitro with the Stratagene mCAP mRNA Capping Kit and protocol. The *KAT1* insert was excised from the pSE936 plasmid [S. J. Elledge, J. T. Mulligan, S. W. Ramer, M. Spottswood, R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1731 (1991)] with Xho I and subcloned into Bluescript KS. The plasmid containing the *KAT1* cDNA was linearized with Hind III, and subsequently mRNA was synthesized with T3 RNA polymerase. Oocytes were impaled with two electrodes filled with 3 M KCl, and recordings were made with a Dagan model 8500 voltage clamp amplifier (Dagan Corp., Minneapolis, MN) or a Turbo TEC 01C amplifier in the voltage clamp mode (NPI Electronics, Tamm, Germany). Voltage-pulse protocols, data acquisition, and data analysis were performed with a 33-MHz 386-based microcomputer using the "pClamp" program (Axon Instruments, Foster City, CA). Membrane currents as well as clamped oocyte potentials were recorded on two separate channels for monitoring of time resolution and for later analysis. Current records shown were corrected for leakage with the use of a P/4 correction method [C. M. Armstrong and F. Bezanilla, *J. Gen. Physiol.* **70**, 567 (1977)]. Experiments without leakage subtraction were performed to ensure that a linear background conductance (1.9 ± 1.1 μS; $n = 7$) was subtracted and that the subpulses used for subtraction did not subtract the *KAT1*-induced currents. The voltage range of the subpulses was −60 mV to −85 mV.
15. K^+ Ringer solution contained 115 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $KHCO_3$, 1.0 mM $MgCl_2$, 2mM KOH, and 10.0 mM Hepes (pH 7.4). In the Na^+ Ringer solution, K^+ was replaced by Na^+. To determine the ionic selectivity of KAT1, we perfused the 300-μl recording chamber containing K^+ Ringer solution with at least 10 ml of solution in which K^+ was replaced with Na^+ Ringer solution or 115 mM RbCl, NH_4Cl, CsCl, or LiCl. These solutions also contained 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10.0 mM Hepes (pH 7.4). The 12 mM KCl solution contained 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10.0 mM Hepes (pH 7.4) with sorbitol to balance the osmotic strength equal to that of the K^+ Ringer solution.
16. N. Dascal, *Crit. Rev. Biochem.* **22**, 317 (1987).
17. S. Hagiwara, S. Miyazaki, W. Moody, J. Patlak, *J. Physiol.* **279**, 167 (1978); M. Lindau and J. M. Fernandez, *J. Gen. Physiol.* **88**, 349 (1986); M. R. Blatt, *ibid.* **99**, 615 (1992).
18. For single-channel recordings, oocytes were bathed in a solution with an osmolality of 450 mmol kg^{-1} for approximately 30 min or until the vitelline membrane was visually detached from the oocyte plasma membrane [C. Methfessel *et al.*, *Pfluegers Arch.* **407**, 577 (1986)]. The vitelline membrane was carefully removed with a pair of forceps. Oocytes were then perfused with Na^+ Ringer solution and allowed to settle and stick to the glass at the bottom of the recording chamber. The resting potential of oocytes after removal of the vitelline membrane was in the range of 0 to −10 mV. Recordings were made with Na^+ Ringer solution in the bath and K^+ Ringer solution in the pipette (*15*). Pipettes were pulled from Kimax-51 capillary glass (Kimble Products, Owens, IL) as described (C. Methfessel *et al.*, *ibid.*, p. 577). Single-channel recordings were performed with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA) and filtered at 2 kHz before pulse code modulation and storage on videotape. Data were subsequently filtered at 500 Hz with 8-pole Bessel characteristics, digitized with the data acquisition program "Fetchex," and analyzed with "Fetchan" (Axon Instruments).
19. D. P. Schachtman, J. I. Schroeder, W. J. Lucas, J. A. Anderson, R. F. Gaber, unpublished results.
20. J. I. Schroeder and H. H. Fang, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11583 (1991).
21. Y. Cao, M. Anderova, N. M. Crawford, J. I. Schroeder, *Plant Cell* **4**, 961 (1992).
22. E. Epstein, *Mineral Nutrition in Plants: Principles and Perspectives* (Wiley, New York, 1972); I. W. Newman, L. V. Kochian, M. A. Grusak, W. J. Lucas, *Plant Physiol.* **84**, 1177 (1987); L. V. Kochian and W. J. Lucas, in *Advances in Botanical Research*, J. A. Callow, Ed. (Academic Press, London, 1988), vol. 15, p. 93.
23. C. L. Slayman, *J. Bioenerg. Biomembr.* **19**, 1 (1987); M. R. Sussman and J. F. Harper, *Plant Cell* **1**, 953 (1989).
24. R. J. Haynes and K. M. Goh, *Biol. Rev.* **53**, 465 (1978).
25. A. J. Bloom and J. Finazzo, *Plant Physiol.* **81**, 67 (1986).
26. L. Y. Jan and Y. N. Jan, *Nature* **345**, 672 (1990).

27. M. Noda *et al.*, *ibid.* **312**, 121 (1984); R. E. Greenblatt, Y. Blatt, M. Montal, *FEBS Lett.* **193**, 125 (1985); H. R. Guy and P. Seetharamulu, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 508 (1986); S. R. Durell and H. R. Guy, *Biophys. J.* **62**, 238 (1992).
28. O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfluegers Arch.* **391**, 85 (1981).
29. We thank M. Anderova [University of California, San Diego (UCSD)] for assistance with oocyte isolation; W. Gassmann (UCSD) for assistance with some single-channel recordings; J. Newport (UCSD) for use of a *Xenopus* colony; Y. Cao (UCSD) for advice on mRNA preparation; M. Montal (UCSD) for use of the Turbo TEC amplifier for some voltage clamp recordings; and L. V. Kochian, M. Montal, N. Crawford, J. Ward, and V. Dionne for discussions and comments on the manuscript. Supported in part by USDA grant 90-37261-5411 (to L. V. Kochian, W.J.L., and R.F.G.); NSF grant DCB8711346 (to R.F.G.); NSF Postdoctoral Fellowship in Plant Biology (to D.P.S.); a grant from the Powell Foundation; National Science Foundation grant DCB9004977; and NSF Presidential Young Investigator Award (to J.I.S.).

2 June 1992; accepted 14 September 1992

Thermal Stability Comparison of Purified Empty and Peptide-Filled Forms of a Class I MHC Molecule

Margaret L. Fahnestock, Ilana Tamir, Linda Narhi, Pamela J. Bjorkman*

A secreted form of a class I major histocompatibility complex (MHC) molecule was denatured and renatured in vitro in the absence of peptide. The resulting empty class I heterodimer was immunologically reactive and structurally similar to a heterodimer renatured in the presence of an appropriate restricted peptide. Thermal stability profiles indicated that the two forms of heterodimer differed in their resistance to denaturation by heat but that a significant portion of the empty class I heterodimers had a native conformation at physiological temperatures. Free energies calculated from these data gave a direct measure of the stabilization of the class I MHC molecule that resulted from peptide binding.

Class I MHC molecules bind short peptides derived from intracellular proteins that are transported along with the MHC molecule to the cell surface, where the complex is recognized by the antigen-specific receptor on a T cell (*1*). For most alleles, folding and surface expression of the class I heavy chain is dependent on the presence of its associated β_2-microglobulin (β_2M) light chain. Experiments in a mutant cell line in which class I surface expression was rescued by extracellular addition of peptide were initially interpreted to suggest that the peptide is required for proper folding and assembly of class I polypeptide chains; the peptide was hypothesized to act as a scaffold, without which the native class I structure could not form (*2*). However, empty class I molecules are assembled and expressed in mutant and nonmutant cell lines in the absence of added peptides (*3*, *4*) and are readily detectable on the cell surface if cells are grown at 26°C (*4*). At 37°C, empty class I molecules also reach the cell surface but rapidly become undetectable by conformationally sensitive antibodies unless stabilized by the binding of an exogenously added peptide or by the addition of excess β_2M (*4*, *5*). Although empty class I molecules are reported to be unstable at physiological temperatures (*4*, *5*), the thermodynamic stability of a purified empty class I molecule has not been directly compared with the same molecule in its peptide-filled form. Here, we compare the thermal denaturation profiles of the murine H-2K^d molecule, assembled in vitro from separated heavy and light chains in the presence and absence of a synthetic peptide, to K^d occupied with an endogenous mixture of peptides. From these data, we calculated the free energy contributed by the peptide to the stabilization of the K^d heterodimer and evaluated the portion of empty molecules that were folded at physiological temperatures. The stability assay used is a method for evaluating peptide binding to purified MHC molecules and can be used to compare the degree of stabilization conferred by peptides of different compositions and sizes.

A secreted form of K^d was efficiently expressed in Chinese hamster ovary (CHO) cells with a glutamine synthetase–based amplifiable expression system (*6*). A stop codon was inserted into the cDNA encoding the heavy chain of K^d after amino acid 284 by use of the polymerase chain reaction (*7*). The resulting modified cDNA was subcloned into an expression vector (*8*) that carried the glutamine synthetase gene as a selectable marker and as a means of gene amplification in the presence of the drug methionine sulfoximine (MSX) (*6*). The cDNAs encoding human or murine β_2M were subcloned into a similar expression vector (*8*) that lacked the glutamine synthetase gene. The heavy chain expression plasmid was transfected into CHO cells either alone or in combination with one of the β_2M expression plasmids, and cell lines resistant to high concentrations of MSX were selected. Supernatants from these CHO clones were tested for the presence of secreted K^d heterodimers by an enzyme-linked immunosorbent assay (ELISA) with antibodies against both heavy and light chains (*9*); the results were verified by immunoprecipitation with a K^d-specific monoclonal antibody (MAb) (*9*). The lines expressing the most K^d were those that had been transfected with the K^d and human β_2M combination (10 to 25 mg/liter), with undetectable amounts of heavy chain secreted in the absence of transfected β_2M cDNA and intermediate amounts detected in cells transfected with the K^d and murine β_2M combination (1 to 2 mg/liter). The increased recovery of the hybrid class I heterodimer over the completely murine heterodimer may reflect the greater stability reported for murine class I heavy chains that are complexed with human rather than with murine β_2M (*10*).

The highest expressing clone (K^d heavy chain and human β_2M combination) was introduced into a hollow fiber bioreactor device (*11*) in the presence of 100 μM MSX. Supernatants containing K^d were harvested daily and contained heterodimer up to 100 μg/ml, as quantitated by ELISA (*9*). Heterodimer was isolated from culture supernatants by immunoaffinity chromatography (*12*). Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) of purified K^d showed several species migrating between 44 and 45 kD, corresponding to truncated heavy chain, and a sharp band at 12 kD, corresponding to β_2M. Digestion of purified K^d with peptide-*N*-glycosidase F (PNGase F) had no effect on the lower band but converted the upper band to a single sharp band migrating at 32 kD, the expected size of the 284–amino acid, truncated heavy chain, which indicates that the observed heterogeneity results from extensive N-linked glycosylation (Fig. 1). The complex eluted from a gel filtration column as a single species of 60 to 63 kD (*13*). NH_2-terminal sequencing of purified K^d yielded sequences in equimolar amounts that correspond to the first 20 residues of the K^d heavy chain and human β_2M (*13*). Hamster β_2M (*14*) and bovine β_2M sequences (*15*) were undetectable, which suggests that association with endogenous hamster β_2M and exchange with bovine β_2M in the medium (*16*) were minimal. Peptides associated with the hybrid heterodimer were isolated by acid elution (*17*) and sequenced. Tyrosine and proline predominated in the second and fourth posi-

M. L. Fahnestock, I. Tamir, P. J. Bjorkman, Division of Biology and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125. L. Narhi, Biophysics, Amgen, Thousand Oaks, CA 91320.

*To whom correspondence should be addressed.