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Cloning and characterization of a potassium-coupled amino acid transporter

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ABSTRACT **Active solute uptake in bacteria, fungi, plants, and animals is known to be mediated by cotransporters that are driven by Na^+ or H^+ gradients. The present work extends the Na^+ and H^+ dogma by including the H^+ and K^+ paradigm. Lepidopteran insect larvae have a high K^+ and a low Na^+ content, and their midgut cells lack Na^+/K^+ ATPase. Instead, an H^+ translocating, vacuolar-type ATPase generates a voltage of approximately −240 mV across the apical plasma membrane of so-called goblet cells, which drives H^+ back into the cells in exchange for K^+, resulting in net K^+ secretion into the lumen. The resulting inwardly directed K^+ electrochemical gradient serves as a driving force for active amino acid uptake into adjacent columnar cells. By using expression cloning with *Xenopus laevis* oocytes, we have isolated a cDNA that encodes a K^+-coupled amino acid transporter (KAAT1). We have cloned this protein from a larval lepidopteran midgut (*Manduca sexta*) cDNA library. KAAT1 is expressed in absorptive columnar cells of the midgut and in labial glands. When expressed in *Xenopus* oocytes, KAAT1 induced electrogenic transport of neutral amino acids but excludes α-(methylamino)isobutyric acid and charged amino acids resembling the mammalian system B. K^+, Na^+, and to a lesser extent Li^+ were accepted as cotransported ions, but K^+ is the principal cation, by far, in living caterpillars. Moreover, uptake was Cl^--dependent, and the K^+/Na^+ selectivity increased with hyperpolarization of oocytes, reflecting the increased K^+/Na^+ selectivity with hyperpolarization observed in midgut tissue. KAAT1 has 634 amino acid residues with 12 putative membrane spanning domains and shows a low level of identity with members of the Na^+ and Cl^--coupled neurotransmitter transporter family.**

To remain alive all cells actively accumulate nutrients across their cell membrane. Since Skou (1) isolated the Na^+/K^+ ATPase and Crane (2) showed that sugar transport into mammalian intestinal epithelial cells is driven by Na^+ glucose cotransport, the dogma has arisen that solute transport into cells occurs via cotransporters that are driven by Na^+ gradients in animals and Na^+ or H^+ gradients in bacteria, fungi, and plants. This dogma was strengthened when the first cDNA encoding a Na^+-coupled glucose cotransporter, SGLT1 from rabbit small intestine, was cloned (3). Now the Na^+ dogma is being challenged by validation of the H^+ and K^+ paradigm. In the much-studied tobacco hornworm, *Manduca sexta*, Na^+ concentrations are very low and Na^+/K^+ ATPase is not detectable in midgut cells. Instead, a voltage of ≈−240 mV, generated by an H^+ vacuolar type ATPase (V-ATPase) (4, 5), secondarily drives exchange (antiport) of K^+ for $2H^+$, resulting in an inwardly directed electrochemical K^+-gradient (Fig. 1*a*), which serves as a driving force for active amino acid uptake into columnar cells (6). The well-characterized K^+-coupled amino acid uptake across an H^+ V-ATPase energized membrane, in cells that lack an Na^+/K^+ ATPase (6), constitutes an important alternative to the Na^+ dogma. Mammalian and bacterial cells commonly use Na^+ or H^+ gradients to drive uptake of polar solutes across the apolar, lipid barrier via transporter proteins. In far more widely distributed insects and fresh-water animals Na^+ is scarce and, in many cases, H^+ translocating V-ATPases energize membranes by imposing a transmembrane voltage (6).

Previous *in vitro* studies had revealed that Na^+ can replace K^+ to drive leucine uptake at pH 8.0 in brush border membrane vesicles of *M. sexta* midgut (7). Taking advantage of the Na^+ acceptability, expression cloning of the K^+-coupled amino acid transporter was attempted by measuring labeled leucine uptake in the presence of a Na^+ gradient in *Xenopus* oocytes where an inwardly directed K^+ gradient is not present. This paper reports the cloning and characterization of a unique K^+-coupled amino acid transporter cDNA.

METHODS

Expression Cloning. Total RNA was extracted from fifth instar *M. sexta* larval midgut by the guanidinium isothiocyanate method using cesium trifluoroacetate. Poly(A)$^+$ RNA was isolated and injected into collagenase-treated *Xenopus* oocytes. Rapid filtration tracer uptake measurements were made in pools of 6–8 collagenase-treated oocytes 4 days after injection of 50 ng of poly(A)$^+$ RNA or K^+-coupled amino acid transporter (KAAT1) cRNA. Oocytes were incubated for 1 h in 750 μl of uptake solution (100 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Tris·HCl, pH 8) containing 200 μM ^{14}C-leucine. Uptake of ^{14}C-L-leucine was enhanced in cRNA-injected oocytes as compared with water-injected controls (Fig. 1*b*). The poly(A)$^+$ RNA was size-fractionated by using preparative gel electrophoresis and assayed in oocytes. A directional cDNA library was constructed from the active fraction of poly(A)$^+$ RNA, using the SuperScript Plasmid System (Life Technologies). cDNAs were ligated into the *Not*I and *Sal*I restriction enzyme sites of the expression vector pSPORT1 and electroporated into ElectroMax DH10B cells (Life Technologies). Plasmid DNA was transcribed *in vitro* from pools of 500 clones, and the resulting cRNA was injected into *Xenopus* oocytes.

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Abbreviations: KAAT1, K^+-coupled amino acid transporter; GABA, γ-aminobutyric acid; GAT1, rat GABA transporter.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF006063).
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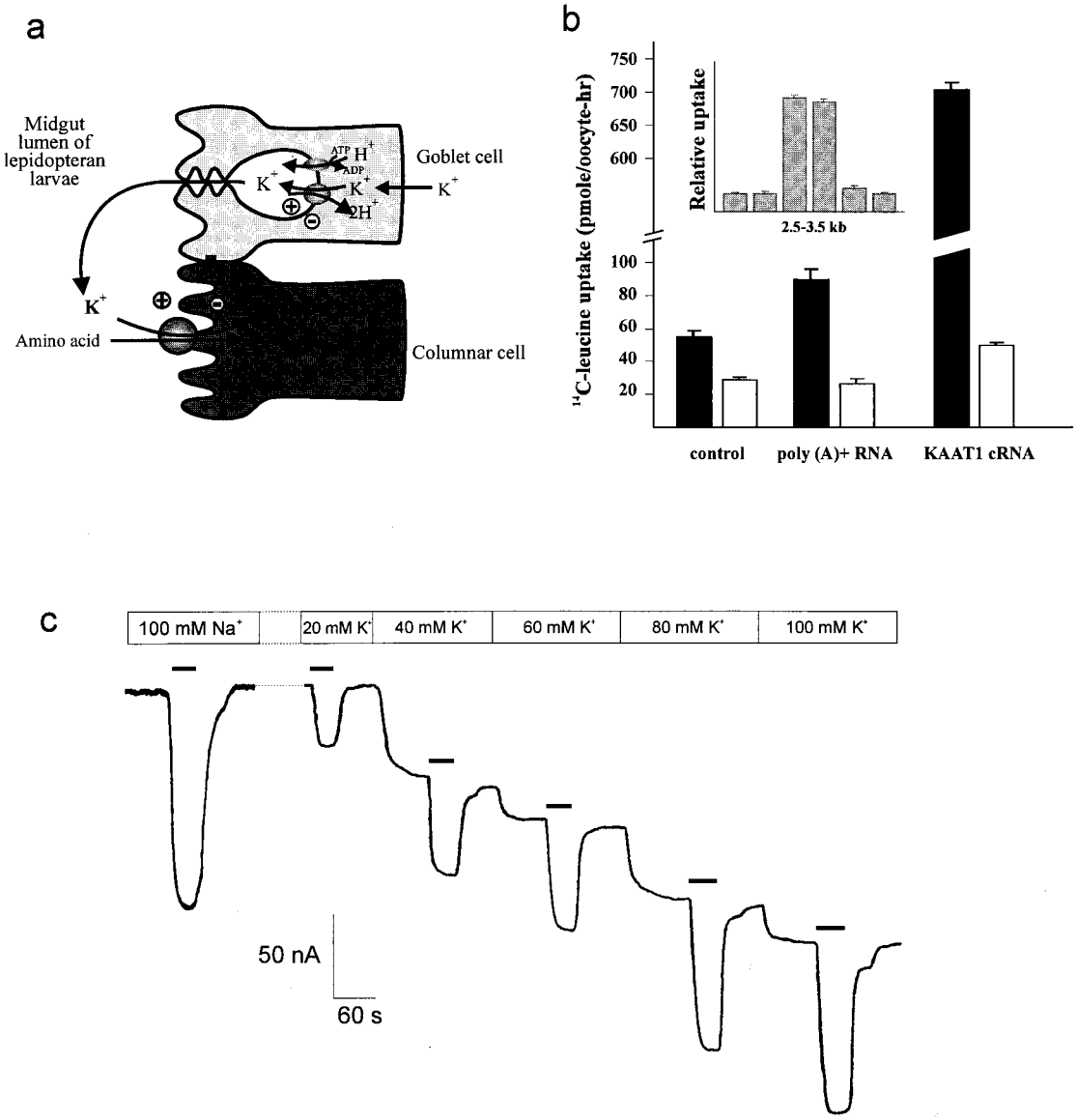

FIG. 1. (*a*) Schematic model of the midgut epithelium of lepidopteran larvae showing the relationship between KAAT1 and other apical membrane components. The apical membrane of goblet cells contains an H^+ vacuolar type ATPase in parallel with a $K^+/2H^+$ antiporter that secretes K^+ into the lumen while maintaining a transapical voltage of ≈ -270 mV. The voltage, attenuated to ≈ -240 mV, appears across the apical (brush border) membrane of electrically coupled columnar cells where it drives K^+-coupled amino acid transport from lumen to cell via KAAT1. (*b*) Na^+-coupled uptake of 200 μM ^{14}C-leucine (1 h) in *Xenopus* oocytes injected with mRNA prepared from *M. sexta* larval midgut or with cRNA synthesized by *in vitro* transcription using KAAT1 cDNA. Open bars indicate water injection. The black bar in the control group indicates injection of total RNA. (*Inset*) Uptake of 200 μM ^{14}C-leucine (1 h) in oocytes injected with size-fractionated poly(A)$^+$ RNA from *M. sexta* larval midgut. All data points represent the mean ± SEM from 6–8 oocytes. (*c*) Leucine (1 mM) evoked uptake currents mediated by KAAT1. A representative oocyte expressing KAAT1 was voltage-clamped at −50 mV and superfused with uptake solution containing 100 mM Na^+ or specified concentrations of K^+. When K^+ was being tested, Na^+ was replaced with choline$^+$. Leucine was applied where indicated by black bars.

Electrophysiological Studies. Stage V–VI oocytes were obtained from mature *Xenopus* females that had been anesthetized with tricaine (1 g/liter). Oocytes were defolliculated by collagenase treatment (1 mg/ml, Boehringer Mannheim) in Ca^{2+}-free Ringer's solution. Capped mRNA was synthesized from linearized plasmids containing the coding region of KAAT1 and approximately 50 ng of RNA in 50 μl of water was injected into the oocytes. The expression was assayed 3–6 days later by two-microelectrode voltage-clamp recording. Data acquisition and analysis used the PCLAMP program suite (Axon Instruments, Foster City, CA). During the two-electrode voltage-clamp recordings, oocytes were first clamped at a holding voltage of −50 mV in the uptake solution containing 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Tris·HCl, pH 7.4 and then perfused with the same solution containing 100 mM KCl instead of NaCl at pH 8.0 in which the experiments were performed (except where otherwise indicated). L-leucine currents reached a steady-state value within 15 s after leucine was introduced into the recording chamber. The voltage then was stepped from the holding value (V_h) to specified experimental voltages at intervals of 1 s. For cation and anion substitution experiments, the solution contained either 100 mM C^+Cl^- or K^+A^- where C^+ is one of the cations and A^- is one of the anions indicated in Fig. 3.

Northern Blot Analysis. Total RNA (10 μg), prepared from fifth instar *M. sexta* larval tissue, was separated on a formaldehyde/agarose gel, blotted onto a nylon filter, and hybridized with full-length KAAT1 cDNA labeled with ^{32}P-dCTP. Hybridization was performed at 42°C in 2× standard saline citrate (SSC)/50% formamide hybridization solution and washed at high stringency (65°C in 0.1× SSC/0.1% SDS).

***In situ* Hybridization.** *In situ* hybridization histochemistry was performed on 7-μm cryosections (postfixed with 4% paraformaldehyde) of fifth instar *M. sexta* larval midgut tissue. Digoxigenin-labeled antisense and sense cRNAs were generated from full-length KAAT1 cDNA by using the Genius Kit (Boehringer-Mannheim) and hydrolyzed to about 200 nts. Sections were hybridized at 65°C for 18 h and washed at the same temperature. The probes were visualized by using anti-digoxigenin Fab fragment and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt/nitroblue tetrazolium chloride as a substrate. The sections were developed after 24 h and photographed.

RESULTS

Expression Cloning of KAAT1 cDNA. Previous *in vitro* studies had revealed that K^+, Na^+, and to a lesser extent Cs^+ are accepted but Rb^+ and Li^+ are rejected as a coupling ion by *M. sexta* brush border membrane vesicles (7). By taking advantage of the Na^+ acceptability, expression cloning of the K^+-coupled amino acid transporter was attempted in *Xenopus* oocytes by measuring labeled leucine uptake in the presence of a Na^+ gradient. Injection into oocytes of poly(A)$^+$ RNA from *M. sexta* larval midgut induced a Na^+-dependent uptake of ^{14}C-L-leucine that was twice as large as that in water-injected controls (Fig. 1*b*), with maximal stimulation in sizes ranging from 2.5 to 3.5 kb (Fig. 1*b*, *Inset*). This fraction was used to construct a cDNA library from which ≈5,000 clones were sequentially screened by expression in oocytes until the cRNA from a single clone that was able to induce leucine uptake 14-fold above control values was isolated.

When leucine was added to oocytes that had been injected with the isolated clone, bathed in standard buffer containing 100 mM NaCl and clamped at −50 mV, a significant inward current was measured with a two-microelectrode voltage-clamp circuit (Fig. 1*c*). Replacement of Na^+ by choline$^+$ in the bathing solution completely abolished the leucine-evoked transport currents (data not shown). More significantly, a large inward current also was induced by leucine perfusion when Na^+ was replaced by K^+; moreover, the amplitude of the current increased with increasing concentrations of external K^+ (Fig. 1*c*). The leucine-evoked inward currents at −50 mV must have been carried solely by K^+, because Na^+ was absent. No currents were evoked by leucine in water-injected control oocytes (data not shown). The clone isolated therefore was designated KAAT1.

Analysis of KAAT1 Amino Acid Sequence. KAAT1 cDNA contained 2,881 nts with an ORF from nucleotides 103-2004 that encodes a 634-aa residue protein of 70 kDa. A search of available databases revealed weak, but significant, identity of KAAT1 with amino acid transporters belonging to the Na^+- and Cl^--dependent γ-aminobutyric acid (GABA) transporter superfamily, with 38% overall identity to rat GABA transporter GAT1 and rat glycine transporter GLYT2 and 37% identity to human brain specific L-proline transporter HPROT (28–30) (Fig. 2*A*). Several critical amino acid residues of the GAT1 GABA transporter, which have been proposed to be involved in Na^+, Cl^-, and substrate recognition, are conserved in the KAAT1 amino acid sequence (Fig. 2) (8–11). Like GAT1, KAAT1 has 12 putative transmembrane domains (Fig. 2*B*).

Functional Characterization of KAAT1. K^+ and leucine uptake kinetics of KAAT1 were determined from leucine-induced steady-state currents after the membrane voltage was stepped from a holding voltage of −50 mV to −150 mV. The dependence on concentration of the currents indicated that the transport is saturable. For potassium ions in 1 mM leucine the K_m was 32 ± 2.8 mM, the V_{max} was 1,256 ± 60 nA, and the Hill coefficient was 1.31 ± 0.23 (all values are mean ± SEM, n = 3) (Fig. 3*a*). For leucine in 100 mM K^+ the K_m was 123 ± 21 μM, the V_{max} was 1,647 ± 371 nA, and the Hill coefficient was 0.99 ± 0.02 (Fig. 3*b*). At the same membrane voltage and leucine concentration, the K_m for Na^+ was 6.0 ± 1.0 mM (data not shown) which is five times lower than that for K^+.

The cation and anion specificity of KAAT1 expressed in oocytes reflected those measured in *M. sexta* brush border membrane vesicles or isolated tissues (7). K^+, Na^+, and to a lesser extent Li^+, but not Rb^+, were accepted as cotransported ions (Fig. 3*c*). Both K^+ and Na^+ were accepted at −50 mV whereas K^+ was preferred over Na^+ at −150 mV, which approaches the physiological value for columnar cells of −240 mV. KAAT1 also appears to be Cl^--dependent because no significant currents were recorded in 100 mM potassium gluconate over the experimental voltage range. The anion selectivity of KAAT1 at −150 mV was $I^- \geq SCN^- \gg Cl^- \gg NO_3^- \approx SO_4^{2-}$ (Fig. 3*d*). The cation and anion-dependence and electronic properties of KAAT1 suggest that the stoichiometry for KAAT1 is two K^+ (or alternatively two Na^+) to one Cl^- to one amino acid.

The substrate specificity of KAAT1 was tested by applying various candidate amino acids under voltage clamp condition. KAAT1 is stereospecific, because L-isomers were preferred. It transports all neutral amino acids tested but not α-(methylamino)isobutyric acid. It rejects L-arginine and L-lysine, which would be cationic at the pH tested; it rejected L-glutamate and even proline. This spectrum is reminiscent of system B in mammalian intestinal and renal epithelia (12) from which no corresponding cDNA has yet been isolated.

KAAT1 mRNA Expression and Tissue Distribution. Northern analysis using full-length KAAT1 cDNA as a probe revealed a strong hybridization signal of 2.9 kb in size in both anterior and posterior regions of the midgut where amino acid occurs, and a weak signal in the labial glands where amino acid uptake may be involved in the secretion of a proteinaceous semiliquid fluid (Fig. 4*a*) (27). *In situ* hybridization was used to determine the cell type in the midgut that expresses KAAT1. Fig. 4 *b* and *c* illustrate that KAAT1 is expressed only in the columnar cells where K^+-coupled amino transport takes place, and no signal was detected in the goblet cells.

DISCUSSION

Amino acid uptake in cells has been investigated for many years. Until now a variety of amino acid transport proteins and corresponding cDNAs have been identified and categorized into many gene families (13). In general, amino acids are transported by coupling to the downhill movement of specific cations, mostly Na^+ and H^+. By using the expression cloning strategy, we report here the cloning of a unique transporter, KAAT1, that couples K^+ rather than Na^+ or H^+ to amino acid absorption in the midgut of lepidopteran *M. sexta* larvae.

K^+-coupled amino acid transport systems have been found in several species of phytophagous lepidopteran larvae (14) whose hemolymph is characterized by a high [K^+] to [Na^+] ratio, in contrast to blood of mammals. Absorption of amino acid in these insects therefore is driven by a K^+ electrochemical gradient maintained by a luminally directed active potassium transporter located at the apical border of goblet cells (6) (see Fig. 1*a*). Characterization of amino acid uptake induced by KAAT1 in *Xenopus* oocytes is in good agreement with the previous physiologic studies in brush border membrane vesicles showing that the expression in amphibian oocytes does not change remarkably the features of this protein.

The kinetic analysis of leucine uptake in the oocytes expressing KAAT1 revealed values that are all in good agreement with those measured in brush border membrane vesicles (7, 15, 16). The K_m for sodium was five times lower than that for potassium. The high affinity of KAAT1 for Na^+ possibly allows the midgut cells to take up this ion even from the low concentrations (<5 mM) found in the lumen of *M. sexta* (17).

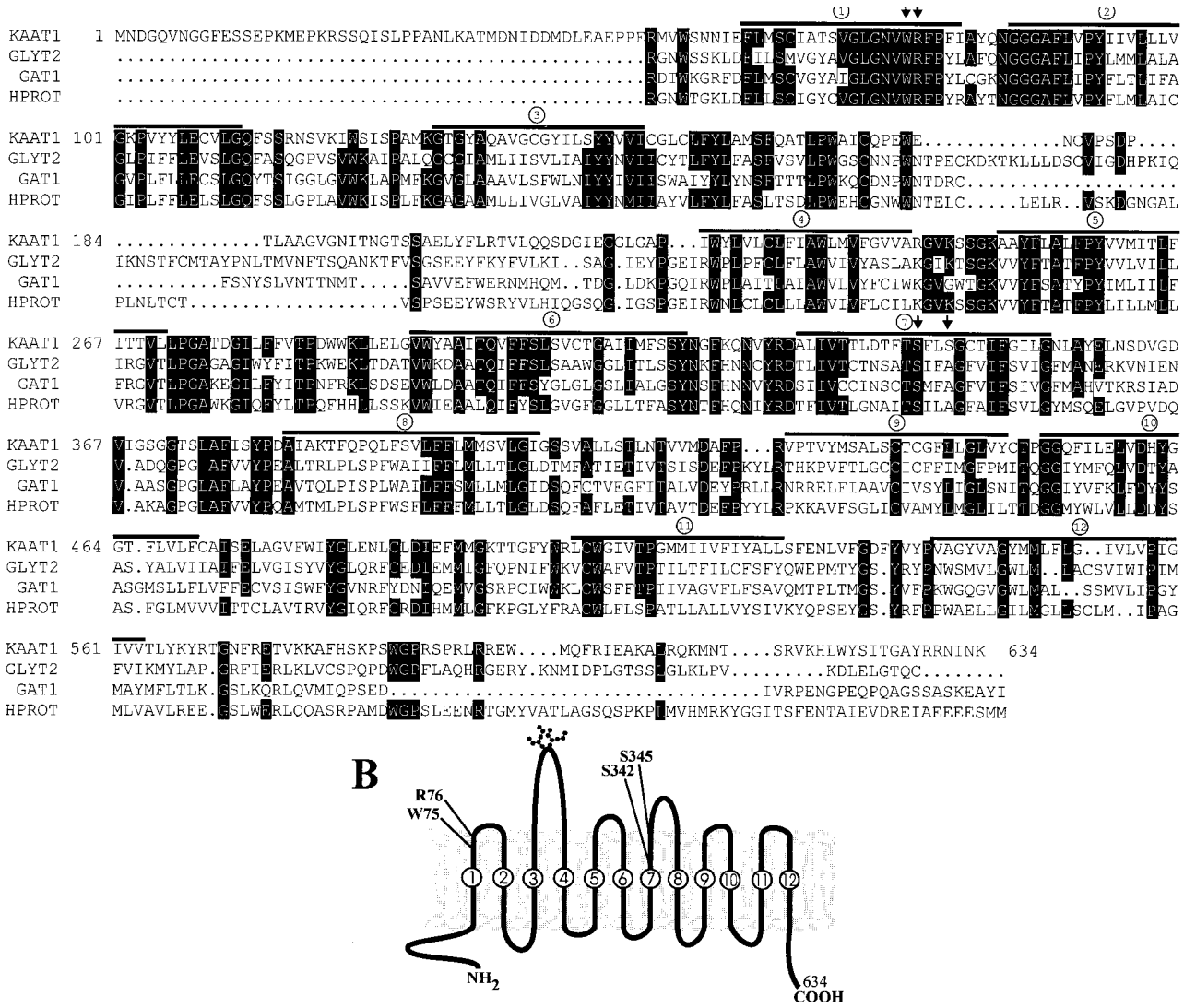

FIG. 2. (*A*) Deduced amino acid sequence of KAAT1 cDNA and alignment with sequences of members of the Na^+-Cl^--dependent GABA transporter family: glycine transporter GLYT2 from rat, GABA transporter GAT1 from rat, and brain specific L-proline transporter (HPROT) from human. Putative transmembrane domains are underlined, and residues considered to be critical for function are highlighted by vertical arrows (see below). (*B*) Hypothetical secondary structure of KAAT1 based on Kyte–Doolittle analysis. Two putative *N*-glycosylation sites are indicated between transmembrane domains 3 and 4. Residues marked are those considered to be functionally important based on the following observations: mutagenesis of GAT1 suggested that tryptophan 75 and arginine 76 (KAAT1 numbering) are involved in Na^+ and Cl^- binding, respectively (25). In the dopamine transporter, it was hypothesized that S342 and S345 (KAAT1 numbering) represent the binding site for dopamine's hydroxyl group (26).

Although it is not yet known how sodium can reach the hemolymph in the absence of a conventional Na^+/K^+ ATPase, Na^+ uptake is essential, because a relatively high Na^+ concentration is present in a special compartment surrounding nerve and muscle cells that operate by a classical Hodgkin–Huxley mechanism (18, 31). Nevertheless, the affinity of the transporter for K^+ and the high concentration of this cation in the gut lumen ensure that the transporter is coupled mainly to K^+ *in vivo*.

The pattern of cation and anion selectivity and the membrane potential dependence of KAAT1 expressed in oocytes are in good agreement with the properties of K^+-coupled amino acid transport systems described for midgut brush border membrane vesicles (7). K^+, Na^+, and to a lesser extent Li^+, but not Rb^+, were accepted as cotransported ions (Fig. 3*c*). K^+ and Na^+ were accepted equally well at -50 mV whereas K^+ was preferred over Na^+ at -150 mV, which approaches the physiological value for columnar cells of -240 mV. Studies addressing the anion dependence of amino acid transport in midgut brush border membrane vesicles did not reveal chloride dependence of transport (7). However, voltage clamp analysis showed that KAAT1 is Cl^- dependent, because replacement of Cl^- by gluconate suppressed the amino acid-evoked currents. This finding suggests a K^+ to Cl^- to amino acid stoichiometry of at least 2:1:1. The Cl^- dependence of KAAT1 indicates that there is not only a structural but also a functional similarity between KAAT1 and Na^+- and Cl^--dependent transporters of the GABA family. However, K^+ coupling appears to be unique to KAAT1. Furthermore, among the tested anions, KAAT1 specificity was: $I^- > SCN^- \gg Cl^- \gg NO_3^- \approx SO_4^{2-}$ (Fig. 3*d*). In particular at hyperpolarized membrane potentials the selectivity for I^- and SCN^- was significantly increased whereas the pattern for other anions was not changed (Fig. 3*d*). Overall these observations suggest that the membrane potential is not only the driving force of the K^+-dependent amino acid transport but it also influences the properties of KAAT1.

Inhibition and countertransport studies of *M. sexta* midgut brush border membrane vesicles led to the identification of distinct K^+- coupled transport systems (19–22). The substrate

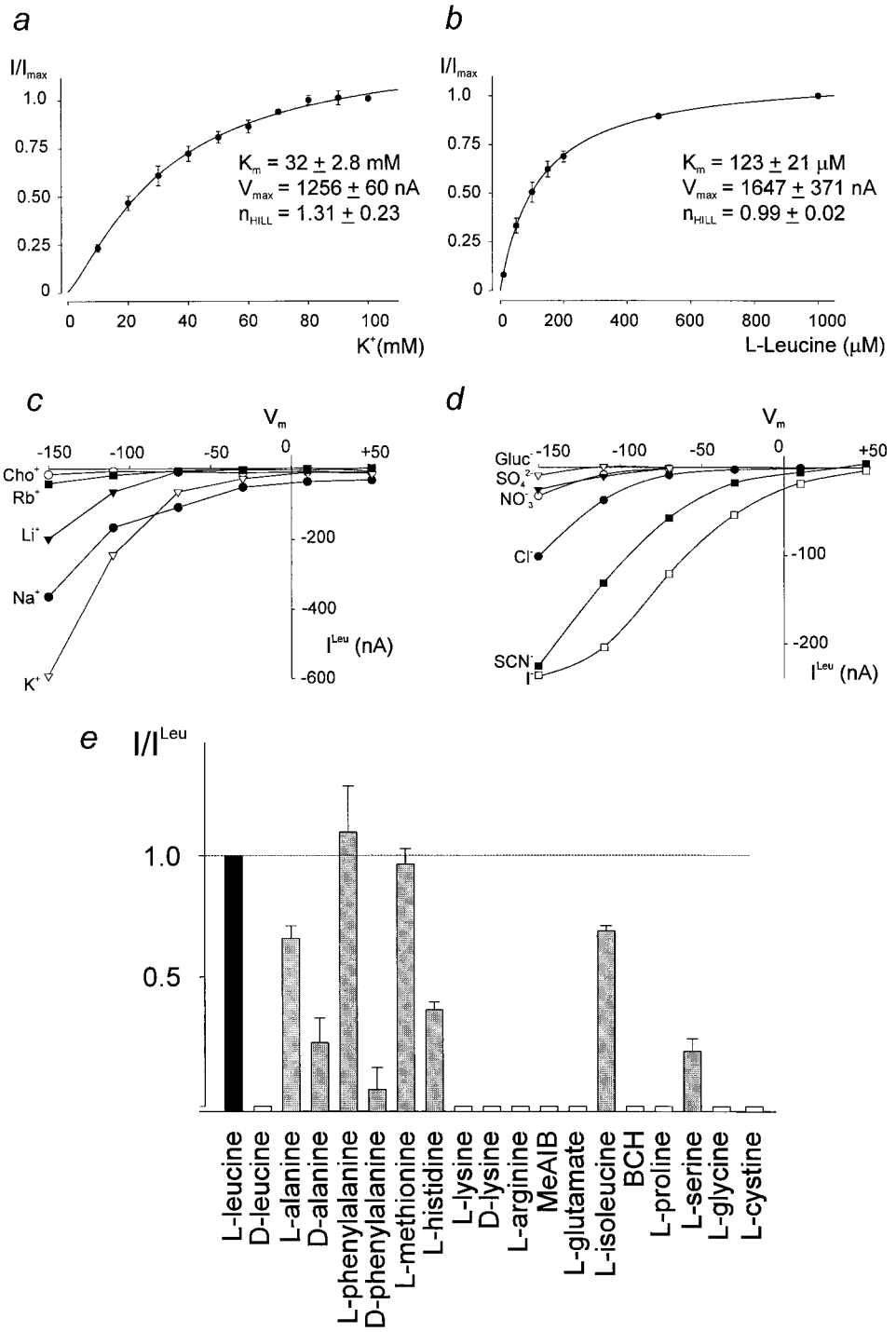

FIG. 3. (*a* and *b*) External K^+ and leucine concentration dependence of KAAT1-evoked uptake currents. Data are steady-state currents obtained at −150 mV by using a voltage jump protocol that steps the membrane potential to −150 mV for 100 ms from a holding potential of −50 mV. Data points (mean ± SEM, $n = 3$) were normalized against the maximal current from the same oocyte and fitted by least-squares according to the equation: $I/I_{max} = [S]_n/\{[S]_n + K^n_{0.5}\}$. (*c* and *d*) Cation and anion dependency of KAAT1-mediated leucine uptake. The figure shows steady-state current-voltage relations of a representative oocyte between +50 and −150 mV, obtained by subtraction of control currents from the corresponding currents in the presence of 200 μM leucine. (*e*) Substrate specificity of KAAT1. An oocyte expressing KAAT1 was held at −50 mV in the uptake solution containing 100 mM KCl, and specific substrates were applied at 200 μM concentration. Data represent the mean ± SEM of three separate experiments and were normalized against the uptake current induced by 200 μM L-leucine.

specificity of KAAT1 expressed in oocytes corresponds to the B-like neutral amino acid transport system based on the following criteria: KAAT1 is stereospecific, because L-isomers are preferred; it transports all neutral amino acids tested but not α-(methylamino)isobutyric acid; it rejects L-arginine and L-lysine, which would be cationic at the pH tested; it rejected L-glutamate and even proline. This spectrum is also highly reminiscent of system B in mammalian intestinal and renal epithelia (12) from which no corresponding cDNA has yet been isolated.

K^+-coupled amino acid uptake has been reported only in the endoderm-derived midgut and not in the ectoderm-derived, cuticle-lined foregut and hindgut of lepidopteran larvae. KAAT1 mRNA expression followed this same pattern. The

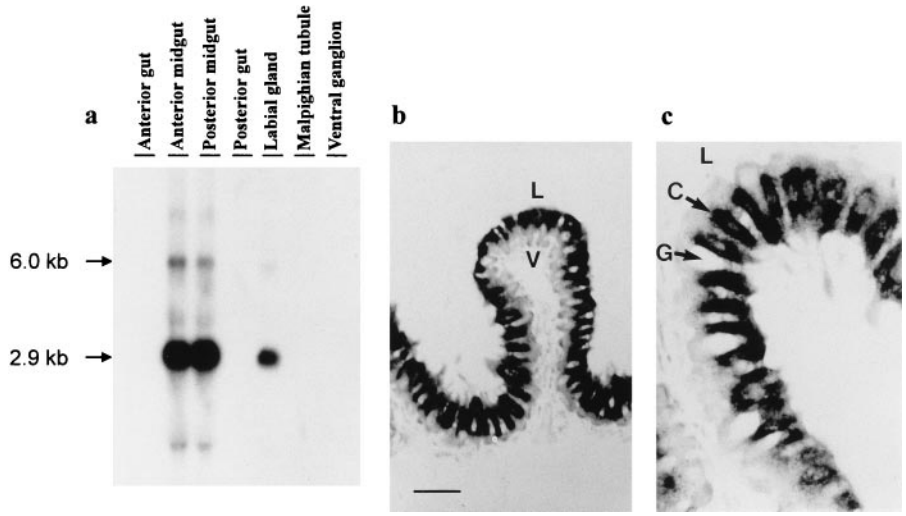

FIG. 4. Tissue distribution of KAAT1 mRNA. (*a*) Northern blot analysis of KAAT1 mRNA expression in *M. sexta* larval tissue. Full-length random primed KAAT1 cDNA was used as a probe. Filter was hybridized at 42°C and final washing was in 0.1× SSC/0.1% SDS at 65°C. (*b*) *In situ* hybridization of *M. sexta* midgut with an antisense KAAT1 cRNA probe revealed intense hybridization signals in epithelial cells lining the lumen (L). V, villus. (Bar, 20 μm.) (*c*) High-power magnification showing that the KAAT1 hybridization signal is found only in columnar cells (C) and not in goblet cells (G).

prominent 2.9-kb band was present in both anterior and posterior regions of the midgut but not in foregut or hindgut. *In situ* hybridization indicated that KAAT1 was expressed only in the columnar cells where K^+-coupled amino transport takes place. The transcript was not found in the goblet cells, which are responsible for energizing the entire apical membrane and for K^+ secretion into the lumen.

KAAT1 appears to be a new piece in the largely unsolved puzzle of transport protein evolution that may represent an adaptation to the high K^+/Na^+ ratio present in the diet and hemolymph of lepidopteran larvae. The peculiar features of KAAT1 suggest that its inhibitors would represent a new family of caterpillar gut poisons that would spare mammals and could lead to a new class of environmentally safe insecticides. On the other hand, KAAT1 may be closely related to system B of mammalian intestine and kidney. In that case it might be used as a probe to clone this broad scope amino acid transport system from mammalians. An intriguing finding is that KAAT1 has sequence homology with members of the GABA transport family; apparently transporters that mediate neurotransmitter amino acid uptake and those that mediate metabolic amino acid uptake had a common ancestor before their divergence during evolution. The pathway and extent of this divergent evolution can be clarified now that KAAT1 probes are available to hasten the search for new pieces of the evolutionary puzzle. Moreover, structure-function studies of KAAT1 will stimulate the investigation of mammalian K^+-coupled systems, including those of Na, K, 2Cl cotransporters (23), Na^+- and K^+-dependent glutamate transporters (24) and Na, K, ATPases.

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