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MOLECULAR CLONING AND SEQUENCE ANALYSIS OF HUMAN GENOMIC DNA ENCODING A NOVEL MEMBRANE PROTEIN WHICH EXHIBITS A SLOWLY ACTIVATING POTASSIUM CHANNEL ACTIVITY

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SUMMARY: The amino acid sequence for a novel human membrane protein that induces selective potassium permeation by membrane depolarization was deduced by molecular cloning and sequence analysis of its genomic DNA. This protein consists of 129 amino acid residues and shares several structural characteristics with the rat counterpart. These include a single putative transmembrane domain surrounded by many charged amino acid residues, two potential N-glycosylation sites at the amino-terminal portion and a single cysteine residue at the carboxyl-terminal portion. The transmembrane domain and its flanking carboxyl-terminal sequence are highly conserved between the human and rat sequences. Because the slowly activating potassium current elicited by the human protein on its expression in Xenopus oocytes is indistinguishable from that induced by the rat protein, the sequence conserved at the transmembrane domain and its following sequence should play an essential role in the induction of selective K^+ permeation.

Potassium ion channels are essential for operating many cellular functions in both excitable and nonexcitable cells and show a high degree of diversity, varying in their electrophysiological and pharmacological properties (1, 2). In a previous study (3), we reported a novel rat membrane protein that induces selective permeation of potassium ions by membrane polarization. This protein was identified by combining molecular cloning in an RNA expression vector with an electrophysiological assay in Xenopus oocytes. The protein identified consists of 130 amino acids with a single putative transmembrane domain and differs from the known ion channel proteins. The K^+ current induced by this protein is unusually slow in activation and deactivation after electrical polarization. The unique structural and electrophysiological properties of the protein (3), together with its restricted localization in the apical membrane portion of epithelial cells (manuscript in preparation) strongly suggest that it serves as a discrete K^+-conducting ion channel (for convenience, we hereafter refer to it as I_{sK} protein, although it may represent a modulatory protein involved in K^+ permeation (3)).

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The small, simple structure of the I_{sK} protein provides an attractive system to investigate the molecular mechanism involved in induction of the voltage-dependent K^+ channel activity. Since the I_{sK} protein gene was found to be widely distributed in mammals, including the human, comparison of the amino acid sequences of I_{sK} proteins in different species should point out conserved domains within the proteins which should be responsible for the fundamental activity of the I_{sK} protein. This investigation thus concerns the isolation and sequence determination of the human genomic clone encoding the I_{sK} protein. We here report the complete amino acid sequence of human I_{sK} protein and its comparison with the rat counterpart.

EXPERIMENTAL PROCEDURES

Cloning of the Human I_{sK} Protein Gene. Our recent molecular analysis of the cloned rat genomic DNA indicated that the I_{sK} protein gene is not interrupted in the protein-coding region by intron insertion (manuscript in preparation). We therefore chose to isolate genomic clones containing the human I_{sK} protein gene to deduce the amino acid sequence of the human I_{sK} protein. The human λ gene library was kindly provided by Dr. Tom Maniatis (Harvard University). Approximately 6×10^5 λ plaques were screened by hybridization with the 552 base pair (bp) BamHI-KpnI fragment of a rat I_{sK} protein cDNA clone (pKI 27) (3) as described (4). The hybridization and filter washing were carried out at 60°C in 1M NaCl containing 1% sodium dodecyl sulfate and at 60°C in a solution containing 75 mM NaCl, 7.5 mM Na_3 citrate and 0.1% sodium dodecyl sulfate, respectively. 35 hybridization-positive clones were isolated by repeated plaque purification. Since restriction enzyme and blot hybridization analyses indicated that all of these clones contain a common genomic sequence, an approximately 2.5 kbp BamHI fragment that hybridized to the rat I_{sK} protein cDNA was isolated and subcloned into pBluescript KS(+) (clone phKI1) for subsequent experiments. The DNA sequence was determined by the chain termination method (5).

Electrophysiological Measurements. The approximately 900 bp PstI-BamHI fragment covering the I_{sK} protein-coding region was obtained from clone phKI1 and inserted into pBluescript KS(+) (clone phKI2). The PstI and BamHI sites of this fragment were located 23 bp upstream from the predicted translation initiation codon and about 500 bp downstream from the translation termination codon, respectively. After digestion of phKI2 DNA with XbaI, mRNA was synthesized in vitro from this DNA template by T3 RNA polymerase in the presence of the capping nucleotide (3). The mRNA synthesized was injected into oocytes, followed by electrophysiological measurements as described previously (3, 6).

RESULTS

Assignment of the Amino Acid Sequence of Human I_{sK} Protein and Comparison with the Rat Sequence.

Since our recent experiments indicated that the whole protein sequence of rat I_{sK} protein is encoded by a single exon without intron insertion (manuscript in preparation), our attempt to deduce the amino acid sequence of human I_{sK} protein was directed to molecular cloning of

its genomic DNA. We isolated human genomic clones that cross-hybridized to the rat I_{sK} protein cDNA and determined the nucleotide sequence homologous to the rat I_{sK} protein-coding region. Fig. 1 shows the nucleotide sequence of the human I_{sK} protein gene and the amino acid sequence deduced for its coding protein. Two potential translation initiation sites can be predicted from the nucleotide sequence. One is the methionine codon located at the position exactly corresponding to the initiation site of the rat I_{sK} protein mRNA. The other is an additional methionine codon 7-9 nucleotides upstream from the above methionine codon. Because the eukaryotic translation generally begins at the first AUG codon at the 5' end of the mRNA (7), the possibility of the latter initiation site can not be excluded. However, the sequence surrounding the AUG codon is also important in determining initiation efficiency (8), and the sequence surrounding the downstream AUG codon more reasonably agrees with the consensus sequence for the eukaryotic translation initiation site (9) than that surrounding the upstream AUG codon. We thus tentatively assigned the downstream AUG codon as the initiation site of the human I_{sK} protein mRNA. The protein-coding region starting with this initiation site ends at the termination codon, UGA, located at the equivalent position of the rat mRNA and encodes a protein constituting 129 amino acid residues.

Fig. 2 shows the amino acid sequence alignment of human and rat I_{sK} proteins. The human protein is short of one amino acid, when compared

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                                                                  Met Pro Arg
                                                CTGCAGCAGTGGAACCTTA ATG CCC AGG   -1

                    ▼                       10                              20
Met Ile Leu Ser Asn Thr Thr Ala Val Thr Pro Phe Leu Thr Lys Leu Trp Gln Glu Thr
ATG ATC CTG TCT AAC ACC ACA GCG GTG ACG CCC TTT CTG ACC AAG CTG TGG CAG GAG ACA   60

                    ▼                       30                              40
Val Gln Gln Gly Gly Asn Met Ser Gly Leu Ala Arg Arg Ser Pro Arg Ser Ser Asp Gly
GTT CAG CAG GGT GGC AAC ATG TCG GCC CTG GCC CGC AGG TCC CCC CGC AGC AGT GAC GGC  120

                                        50                                      60
Lys Leu Glu Ala Leu Tyr Val Leu Met Val Leu Gly Phe Phe Gly Phe Phe Thr Leu Gly
AAG CTG GAG GCC CTC TAC GTC CTC ATG GTA CTG GGA TTC TTC GGC TTC TTC ACC CTG GGC  180

                                        70                                      80
Ile Met Leu Ser Tyr Ile Arg Ser Lys Lys Leu Glu His Ser Asn Asp Pro Phe Asn Val
ATC ATG CTG AGC TAC ATC CGC TCC AAG AAG CTG GAG CAC TCG AAC GAC CCA TTC AAC GTC  240

                                        90                                     100
Tyr Ile Glu Ser Asp Ala Trp Gln Glu Lys Asp Lys Ala Tyr Val Gln Ala Arg Val Leu
TAC ATC GAG TCC GAT GCC TGG CAA GAG AAG GAC AAG GCC TAT GTC CAG GCC CGG GTC CTG  300

                    *                   110                                 120
Glu Ser Tyr Arg Ser Cys Tyr Val Val Glu Asn His Leu Ala Ile Glu Gln Pro Asn Thr
GAG AGC TAC AGG TCG TGC TAT GTC GTT GAA AAC CAT CTG GCC ATA GAA CAA CCC AAC ACA  360

                                    129
His Leu Pro Glu Thr Lys Pro Ser Pro
CAC CTT CCT GAG ACG AAG CCT TCC CCA TGA ACCCCACCACTGGCTAAA
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Fig. 1. The genomic DNA sequence and the deduced amino acid sequence of human I_{sK} protein. The human genomic clone encoding I_{sK} protein was isolated and sequenced as described under "EXPERIMENTAL PROCEDURES". The amino acid sequence deduced from the genomic DNA sequence is indicated above the nucleotide sequence. The putative transmembrane domain and three amino acids starting from the upstream methionine codon are enclosed by a solid and a dashed line, respectively; arrows, potential N-glycosylation sites; asterisk, a cysteine residue.

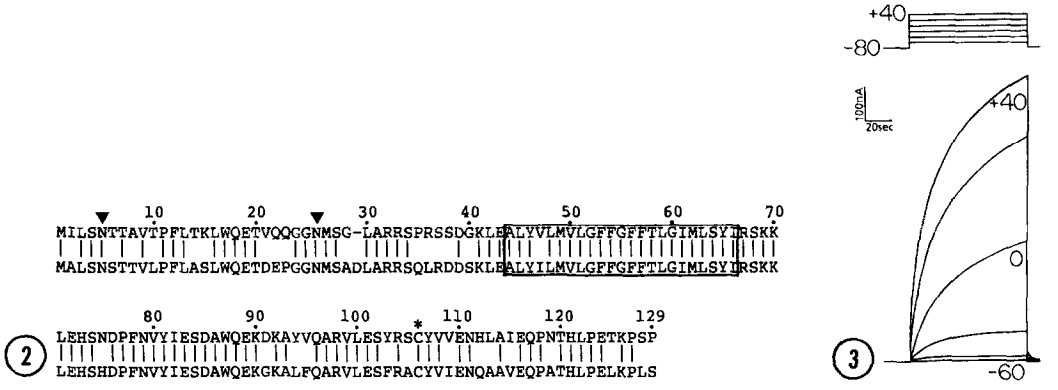

Fig. 2. Comparison of the amino acid sequences of human (upper) and rat (lower) I_{sK} proteins. Bars indicate exact matches between the two amino acid sequences. A hyphen shows a deletion of the amino acid in the human sequence. For other symbols, see Fig. 1.

Fig. 3. Voltage-clamp records of an oocyte injected with the mRNA synthesized from the cloned genomic DNA. The mRNA (4 ng per oocyte) derived from the cloned genomic DNA (phKI2) was injected into an oocyte. After being incubated for 3 days, the oocyte was held at -80 mV, depolarized stepwise to test potentials for 90 sec and then repolarized to -80 mV (upper traces). Currents were measured by conventional two-microelectrode voltage-clamp techniques (lower records). Leakage currents were subtracted from the recording currents by appropriate multiples of the current elicited by a hyperpolarizing pulse to -90 mV.

with the rat counterpart and this occurs between residues 29 and 30. In the previous paper (3), we discussed some structural features characteristic of rat I_{sK} protein and these structural characteristics are further supported by the sequence comparison of the two protein sequences. 1) Human I_{sK} protein contains a distinct hydrophobic segment consisting of 23 continuous uncharged amino acids in its middle portion, thus representing a membrane protein (3). Interestingly, not only this putative transmembrane domain but also its following sequence is extremely homologous between the two sequences: 48 of the 50 amino acid residues between residues 41 and 90 are identical at the corresponding positions. Accordingly, most of the charged amino acid residues surrounding the transmembrane domain are conserved between the human and rat sequences. In contrast, the amino-terminal and carboxyl-terminal portions slightly diverge between the two proteins, and overall, 76% of the amino acid residues are identical at the corresponding positions. 2) There are two potential N-glycosylation sites conforming to the canonical Asn-X-Ser/Thr sequence (10) in the amino-terminal portion of the human sequence, and both of the putative glycosylation sites are present at equivalent positions of the rat sequence. 3) The cysteine residue is conserved in the carboxyl-terminal regions of the two proteins.

Electrophysiological Characterization of Human I_{sK} Protein.

To examine whether the cloned genomic DNA encodes a functional mRNA for I_{sK} protein, we performed a series of electrophysiological experiments. The genomic DNA encoding the protein-coding region was linked to the plasmid T3 promoter and transcribed in vitro with T3 RNA polymerase. The mRNA thus synthesized was injected into Xenopus oocytes, which were then tested for response to membrane polarization. A typical response of an injected oocyte to membrane depolarization is shown in Fig. 3. Depolarization from a holding potential of -80 mV to more positive levels elicited large voltage- and time-dependent outward currents. On repolarization to -80 mV, slow outward tail currents were observed. Currents with similar amplitude and properties were never seen in water-injected control oocytes. Ionic mechanisms underlying outward currents were then examined by determining the current-voltage relationship of the tail current; an oocyte injected with mRNA was held at -80 mV in the external medium containing 2 mM K^+, depolarized to +20 mV for 15 sec, and then repolarized to various potentials. The reversal potential thus obtained was approximately -100 mV (data not shown), which is close to the equilibrium potential of K^+ ions in Xenopus oocytes (11). Furthermore, when the K^+ concentration in the external medium was increased to 20 mM, the reversal potential was shifted to the value expected by the Nernst equation for K^+. Thus, these results indicate that the mRNA generated by transcription of the cloned genomic DNA directs the formation of a functional I_{sK} protein, and that the slowly activating outward current elicited by this protein is carried selectively by K^+ ions.

DISCUSSION

This report describes the nucleotide sequence encoding human I_{sK} protein and the amino acid sequence comparison of the human and rat I_{sK} proteins. Interestingly, while the amino-terminal and carboxyl-terminal regions of the human and rat sequences slightly differ from each other, the putative transmembrane domain and its following region are highly conserved between the two proteins. Because the electrophysiological properties of human I_{sK} protein presented in this paper is indistinguishable from those reported for rat I_{sK} protein (3), the conserved regions should play an essential role in eliciting a K^+ channel activity characteristic of I_{sK} protein. Consistent with this finding, our recent experiments of mutational analysis of rat I_{sK} protein indicated that mutagenic changes of the amino acids immediately following the transmembrane domain markedly affected the K^+ channel activity of I_{sK} protein (manuscript in preparation). Thus, in the light of the sequence

comparison of the human and rat proteins, I_{sK} protein will provide an intriguing system to investigate molecular mechanisms involved in voltage-dependent, selective K^+ permeation.

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