Molecular cloning of a widely expressed human homologue for the *Drosophila trp* gene

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Abstract **The *Drosophila* transient receptor potential (*trp*) gene and its homologue, *trpl*, have been suggested to mediate calcium entry during the insect's phototransduction process. We isolated a human cDNA, human *trp*-1 (*Htrp*-1), encoding a polypeptide of 793 amino acids that is 37% identical (62% similar) to *Drosophila trp* and *trpl*. Northern analysis showed that the *Htrp*-1 transcript is approximately 5.5 kb and expressed in most human tissues, with higher amounts in ovary, testis, heart, and brain. Isolation of *Htrp*-1 suggests that a *trp*-type protein is present in mammals and should provide a useful tool in studying calcium-depletion induced calcium influx processes.**

Key words: Transient receptor potential; Calcium influx channel; Ca^{2+} signaling

1. Introduction

Calcium regulation plays an important role in many cellular processes. In non-excitable mammalian cells, activation of phosphoinositide-specific phospholipase C (PLC) produces inositol 1,4,5-trisphosphate (IP_3), which in turn causes the release of intracellular calcium from its storage pools in the endoplasmic reticulum. This results in a transient elevation of cytosolic-free Ca^{2+}, which is normally followed by a Ca^{2+} influx from the extracellular space. By refilling the pools, Ca^{2+} influx plays an important role in prolonging the Ca^{2+} signal, allowing for localized signaling, and maintaining Ca^{2+} oscillations [1].

Calcium influx in non-excitable cells is thought to occur through plasma membrane channels which, in contrast to the voltage-dependent Ca^{2+} channels in excitable cells, are operated not by changes of membrane potentials but rather by how full the internal Ca^{2+} stores are [2]. Although studies using either fluorescent Ca^{2+} indicators or electrophysiological techniques have suggested that multiple types of Ca^{2+} permeant channels may be involved in different cell types to fulfill the influx function, the molecular structure of the channels and the mechanism that regulates the influx have remained unclear and represent one of the major unanswered questions of cellular Ca^{2+} homeostasis [3–5].

Candidates involved in voltage-independent Ca^{2+} entry into cells include a gene product missing in a *Drosophila* mutant, the transient receptor potential (*trp*), and its homologue, *trp*-like (*trpl*). The insect phototransduction pathway is mediated through the activation of PLC coupled by a G_q type protein [6]. The consequent generation of IP_3 and the release of Ca^{2+} from its intracellular storage pools is believed to lead to the opening of a light sensitive ion channel and generation of a depolarizing receptor potential. Similar to intracellular Ca^{2+} changes in mammalian cells following stimulation by agonists acting via PLC, electroretinograms of *Drosophila* eyes are biphasic with an initial peak followed by a sustained phase of which the latter is dependent on extracellular Ca^{2+}. This sustained phase is absent in the *trp* mutant which was therefore proposed to be caused by a defect in the Ca^{2+} influx pathway [6]. The *trp* gene was cloned [7,8]. Subsequently, molecular cloning of a *Drosophila* calmodulin binding protein showed it to be a homologue of the *trp* gene product and named *trp*-like or *trpl* [9]. A detailed analysis of the *trpl* sequence showed that it shares moderate homology with voltage-dependent Ca^{2+} and Na^+ channels at their putative transmembrane regions. However, in clear contrast with the voltage-dependent channels, it lacks the positively charged amino acid residues at the presumed S4 segment which are thought to act as voltage sensors that promote gating in response to changes in membrane potentials. The structural homology to Ca^{2+} and Na^+ channels together with the absence of charged residues in *trpl* and *trp* suggested that these proteins may form voltage-independent ion channels. This was demonstrated recently by expression of the cDNAs for *trp* and *trpl* in insect *Sf*9 cells using the baculovirus system. It was fount that *trp* forms a Ca^{2+} permeable cation channel which is activated by store depletion with thapsigargin [10] whereas *trpl* forms a Ca^{2+} permeable non-selective cation channel which is not only constitutively active when over-expressed in *Sf*9 cells but also can be up-regulated by receptor stimulation [11–13]. However, it was also noticed that neither *trp* nor *trpl* mimicked the endogenous Ca^{2+} influx channel of the *Sf*9 cells, suggesting the existence of at least one other channel in insects involved in Ca^{2+} entry [10].

Because of the similarities between the IP_3/Ca^{2+} pathway in mammalian tissues and the phototransduction process in insects, it is expected that homologous genes of *trp* and/or *trpl* should exist in mammalian tissues, which may form one or several of the cation channels that participate in Ca^{2+} entry following receptor stimulation. In this article, we report the isolation of a human gene (human *trp*-1, or *Htrp*-1), a homologue of *Drosophila trp* and *trpl*. This gene is widely expressed in most human tissues.

2. Materials and methods

2.1. Isolation and sequencing of cDNA clones

We used a synthetic 45 nucleotide long oligonucleotide sequence, 5′-TTGAACATAAATTGCGTAGATGTGCTTGGGAGAAATGC-

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no. U31110.

Abbreviations: EST, expressed sequence tag; IP_3, inositol 1,4,5-trisphosphate; PLC phosphoinositide-specific phospholipase C; *trp*, transient receptor potential; *trpl*, *trp*-like; *Htrp*-1, human *trp*-1.

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TGTTACC-3′, labeled at the 5′-end with ^{32}P by incubating with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase to screen a λgt10 human kidney cDNA library using standard protocols as described [14]. Hybridization was carried out in a shaking waterbath at 65°C overnight. The filters were washed at 65°C with 2 × SSC/0.1% SDS (1 × SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0). One positive clone was obtained from this library containing an insert of 1.5 kb with multiple *Eco*RI sites. The *Eco*RI fragments were subcloned into plasmid Bluescript KS(+) and sequenced. One 0.67 kb *Eco*RI fragment was later used as a probe for subsequent screening of other human cDNA libraries after labeling with [α-^{32}P]dCTP using the Klenow enzyme and random hexamers [15].

A primer specific library was constructed to facilitate the cloning of the N-terminal region of the *Htrp*-1 gene. Poly(A) RNA was prepared from 2.5×10^8 from human embryonic kidney cells, HEK 293, using an mRNA isolation kit from Collaborative Biomedical Products (Bedford, MA, USA). Complementary DNA was synthesized, using a cDNA Synthesis module from Amersham, starting with 5 μg of the mRNA and a mixture of the following oligonucleotide primers: 5′-TCGCACGCCAGCAAGAAAAG-3′, 5′-CGATGAGCAGCTAAAATGAC-3′, and 5′-TGTCAGTCCAATTGTGAAAGA-3′, each at the final concentration of 1.4 μM. A λgt10 library was constructed using Amersham cDNA cloning kits following manufacturer's protocols.

DNA inserts were sequenced by the dideoxynucleotide termination method using [α-^{35}S]dATP and Sequenase version 2.0 (United States Biochemicals) as previously described [15]. The sequence was confirmed by sequencing both strands using double-stranded plasmids as templates and either universal primers or *Htrp*-1 specific synthetic oligonucleotides as primers. Other standard nucleic acid and bacteriological manipulations were performed as described [14].

2.2. Database searches and sequence analysis

Protein and nucleic acid searches were performed using the BLAST network service of the National Center for Biotechnology Information via an E-mail server. DNA fragment assembly, restriction mapping, protein hydropathy analysis and alignment and all other sequence-dependent analyses were performed using the Wisconsin Sequence Analysis Package from the Genetics Computer Group (GCG).

2.3. Northern analysis

Human multiple tissue Northern blots (Clontech) were prehybridized in a Rapid-hyb buffer (Amersham) at 60°C for 2 h and then hybridized in the same buffer with ^{32}P-labeled cDNA probe (4×10^6 cpm/ml) at 60°C for 14 h. After rinsing with 2 × SSC/0.05% SDS, the filters were washed twice in the same solution and then twice in 0.2 × SSC/0.1% SDS at 60°C. The filters were exposed to X-ray film at −70°C with intensifying screens for desired periods of time. The probe for *Htrp* was made from the 0.67 kb *Eco*RI fragment of the *Htrp*-1 cDNA and a control probe was a human cDNA for β-actin. Both probes were labeled by random prime labeling with [α-^{32}P]dCTP.

3. Results

Expressed sequence tags (EST) are partial, 'single-pass' cDNA sequences deposited in the Genbank database. Many of these sequences are homologous to proteins from other organisms and many of them may contain protein-coding regions that represent novel gene families [16]. We reasoned that such a cDNA sequence encoding a mammalian homologue for the *trp* gene might exist in the database. Therefore, we used the deduced amino acid sequence of the *Drosophila trp* as a query to search the Genbank database using TBLASTN, a program that allows comparison of a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames. A human EST (EST05093) was found to encode an amino acid sequence that shares similarity with the *Drosophila trp* sequence from Glu^{33} to Asn^{80} as follows:

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Drosophila trp:     EKNFILSCERGDLPGVKKILEEYQGTDKFNINCTDPMNRSALISAIEN
                    || | | | ||  |||||||   | ||||  | | |  |||
Deduced EST05093:   EKLFLLACDKGDYYMVKKILEENSSGD.LNINCVDVLGRNAVTITIEN
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The 297 nucleotide sequence of this EST was determined from a cDNA clone isolated from a fetal human brain cDNA library and was deposited in GenBank by Adams et al. [16]. The deduced peptide sequence of EST05093 was then compared with the protein sequences of the *Drosophila trpl* and a *C. elegans trp* homologue (ZC21.2, Genbank accession #L16685). This revealed that the C-terminal region of the EST peptide is homologous to the N-terminal regions of all the *trp*-type proteins. We thus synthesized an oligonucleotide according to the 3′ region of the EST05093 and used it as a probe to screen a human kidney cDNA library. From 1.5×10^6 recombinant phage, we isolated one positive clone, T23. An *Eco*RI digest of the purified λgt10 phage DNA produced three fragments. Among them, a 470 bp fragment hybridized to the oligonucleotide probe used for screening. The sequence of this fragment was determined and found to contain the complete sequence of EST05093. The sequences of the other two *Eco*RI fragments were found to contain open-reading frames which encode amino acid sequences homologous to the *trp* proteins downstream from the region homologous to ETS05093. Thus, T23 appears to be a human *trp* homologue and is tentatively named human *trp*-1 or *Htrp*-1.

A 670 bp *Eco*RI fragment from T23 was then used as a probe to screen other human cDNA libraries, including a λZAP aorta, a λZAP cerebellum, a λgt10 heart and a specifically primed λgt10 library made from oligo(dT)-purified HEK 293 cell mRNA. From all isolated cDNA clones, 13 were sequenced completely. These cDNA clones cover an mRNA of about 5.5 kb, with an open-reading frame of 2379 bases. Comparison of overlapping DNA sequences of clones obtained from kidney, aorta, cerebellum, and heart showed only two silent substitutions of nucleotides which may arise because of polymorphism. Therefore, all the cDNA clones should be the product of the same gene locus.

The open reading frame of the *Htrp*-1 encodes a protein of 793 amino acids. A stop codon is present at 366 bases upstream from the first methionine in the same reading frame. The codon for the second methionine in this sequence, 5′- GCCGCGATGATGGCG-3′, matches better than the first methionine codon the sequence characteristics for translation initiation, i.e. 5′-GCCA/GCCAUGG-3′ as specified by Kozak [17]. Therefore, the translated open reading frame may contain only 792 instead of 793 codons. A more detailed analysis of the cDNA clones indicated that the primary transcript of *Htrp*-1 gene may be spliced in alternative ways. Many of the cDNA clones do not contain a stretch of 102 base pairs which encodes amino acids 109–143. This gives rise to a shorter form of *Htrp-1* with only 759 amino acids.

Searching the Genbank database using BLASTP and the *Htrp-1* protein sequence as a query, we found that only *Droso-*

Table 1
Evolutionary Distances of the *trp* Proteins

	Dtrp	Dtrpl	Ctrp
Htrp-1	124	122	128
Dtrp		78	130
Dtrpl			124

Evolutionary distances were determined using the Kimura protein distance analysis method. The non-conserved regions at the N- and C-termini were not included for calculation of the distances. Abbreviations for *trp* proteins are the same as in Fig. 1.

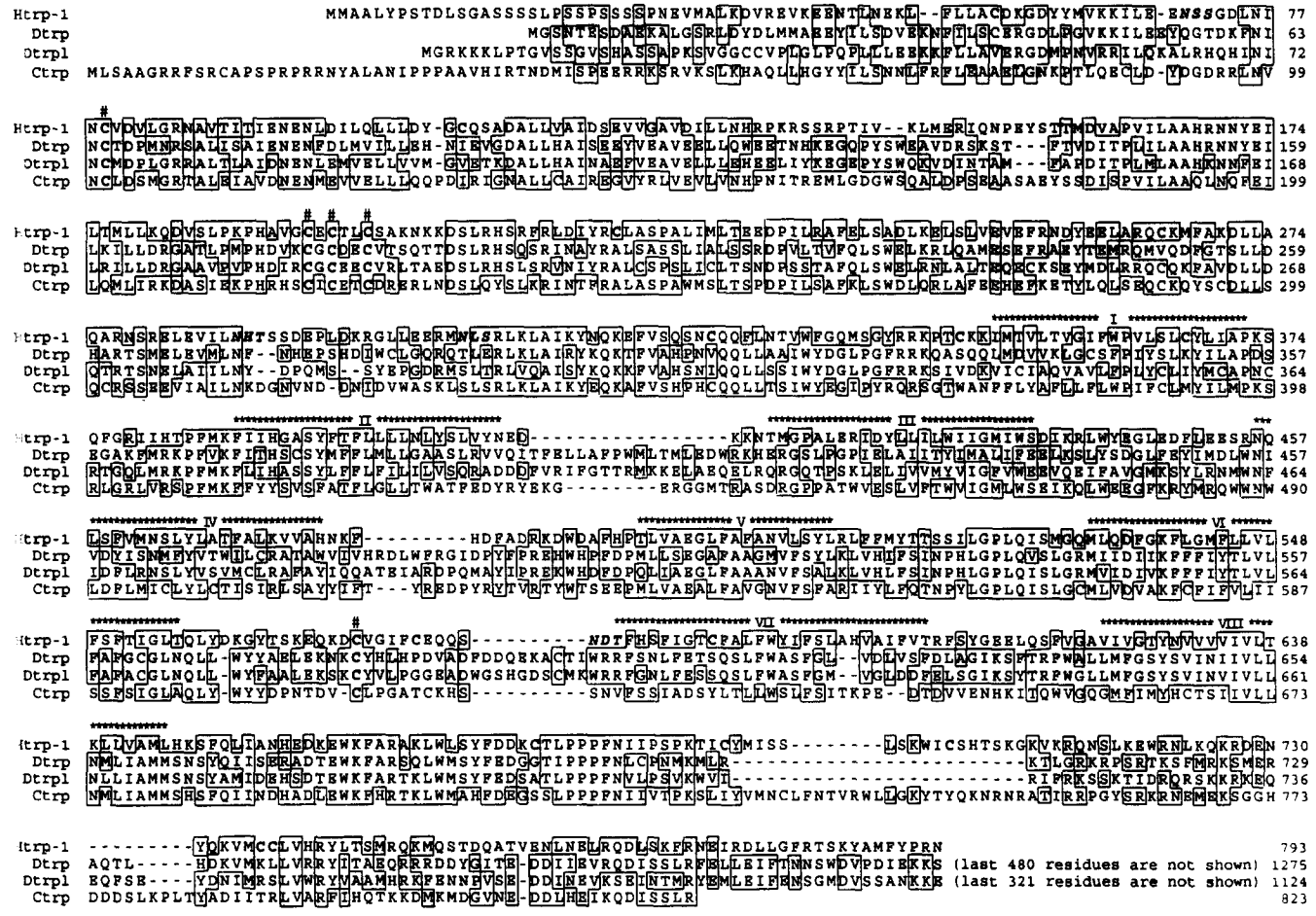

Fig. 1. Amino acid sequence of *Htrp-1* and its alignment with the *Drosophila trp* (Dtrp; *Drosophila trpl* (Dtrpl), and a *C. elegans trp* homologue (Ctrp). Conserved amino acids are boxed. The conserved cysteine residues, which potentially form disulfide bonds for proper protein configurations are marked with '#' above the alignment. The potential glycosylation sites in *Htrp*-1 are indicated in bold italic letters. The hydrophobic regions are labeled in Roman numbers flanking by asterisks above the amino acid sequences.

phila trp, *Drosophila trpl* and *C. elegans trp* have probability scores higher than 300. The remainder of the matched sequences had scores lower than 70. The *Htrp*-1 cloned here is about 37% identical or 62% similar to each of the other three known *trp* proteins. Sequence alignment of all four *trp* proteins shows conserved clusters of short amino acid sequences distributed throughout the entire length of the polypeptides, except that *Htrp*-1 and *C. elegans trp* have much shorter C-termini (Fig. 1). As seen with *Drosophila trp*, *Drosophila trpl* and *C. elegans trp*, hydropathy analysis of the *Htrp*-1 protein (Fig. 2) suggests 8 hydrophobic regions denoted as I through VIII in Fig. 1. These could correspond to transmembrane segments.

The evolutionary distances between each pair of the four *trp* proteins determined by the Kimura method [19] are shown in Table 1.

A Northern analysis using a fragment of *Htrp-1* as a probe shows that a transcript of about 5.5 kb is abundant in human heart, brain, ovary, and testis. Lower amounts of the transcript are also present in many other tissues including, kidney, lung, spleen, pancreas, thymus, skeletal and smooth muscle (Fig. 3). The *Htrp-1* transcript is not detected in human liver mRNA by Northern blotting. However, a mouse *trp*-1 sequence which is 99% homologous to *Htrp*-1 is obtained from mouse liver mRNA by RT-PCR, indicating the presence of *Htrp*-1 in liver mRNA in low amounts.

4. Discussion

We have cloned a novel human gene using an EST derived from human brain. The encoded product of this gene is, in general, much more similar to the products of *trp* and *trpl* of *Drosophila* and a *trp* homologue of *C. elegans* than to anything else present in the GenBank database. The amino acid sequence alignment suggests that the product of this human gene is a

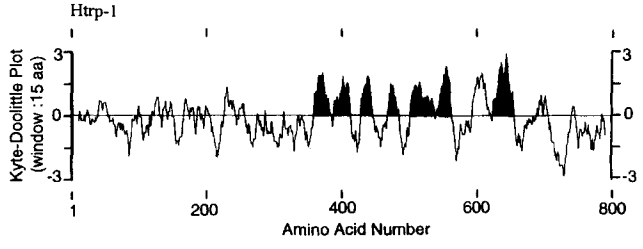

Fig. 2. Hydrophobicity plot of *Htrp*-1. The hydropathy profile was obtained according to the method of Kyte-Doolittle [18] using a window of 15 amino acids.

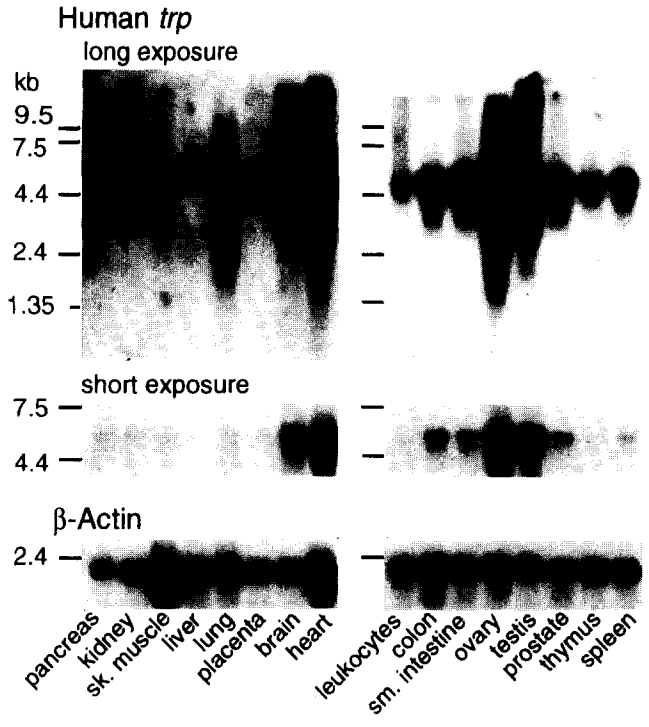

Fig. 3. Expression of *Htrp*-1 mRNA in human tissues. Human multiple tissue mRNA blots were hybridized at 60°C with a labeled probe made from a fragment of *Htrp*-1. Filters were washed at 60°C in 0.2 × SSC and 0.1% SDS. The upper panels show the results using the *Htrp*-1 probe. The middle panels are shorter exposures of the filters from the same hybridization. Lower panels are results of the same filters hybridized with a human β-actin probe.

member of the *trp* gene family, which seems to consist of proteins with relatively conserved N-termini and putative transmembrane regions but more diverse C-terminal tails.

The evolutionary distances between each pair of the four *trp* proteins determined by the Kimura method, as shown in Table 1, are much higher than the distance scores for protein subtypes serving the same function. For example, among 16 different β subunits of voltage-dependent Ca^{2+} channels of various species including the domestic fly and human, the longest distance obtained by comparison using the same method was 72. However, these distances are still lower than for related proteins serving distinct functions. For example, the distance score for the protein sequences of a voltage dependent Ca^{2+} channel and a Na^+ channel is 178, similar to the distances calculated among the *trp* proteins. Therefore, it is possible that each cloned *trp* represents a functionally distinct type of protein. Proteins of the same type, i.e. *trp*, *trpl*, or *Htrp-1*, are yet to be found across different species. Indeed, although the two *Drosophila trp* proteins are most related among the four, the functional roles of *trp* and *trpl* in *Drosophila* phototransduction are thought to be different [6,20]. When expressed in *Sf*9 cells, the *Drosophila trp* was activated by store depletion whereas the *Drosophila trpl* was not and channels formed by these two proteins also had different ion selectivities [10,11]. Part of these differences may result from the fact that at the C-terminus of the *Drosophila trp*, there is a 9-fold repeat of a very hydrophilic eight amino acid sequence (DKDKKPGD), which does not occur in *trpl* and the other two *trp* homologues which have shorter C-terminal tails.

Based on the hydropathy plot, *Htrp*-1 has 8 hydrophobic regions which could potentially form 8 transmembrane segments. However, hydropathy plots of the other *trp* proteins (not shown) indicate that in the *Drosophila* and *C. elagans trp* proteins, the regions that correspond to the 7th hydrophobic region of the *Htrp*-1 are not very hydrophobic. This suggests that in *Htrp*-1, this region may not form a transmembrane segment, if one assumes that the pattern of membrane topology for all *trp* proteins is the same. In addition, as described for the *Drosophila trpl* [9], the regions IV, V, VI, and VIII of the *trp* proteins have some homology with transmembrane segments S3, S4, S5, and S6, respectively, of voltage-gated Ca^{2+} and Na^+ channels (Fig. 4a). This suggests that the *trp* proteins may have similar transmembrane organization as the voltage-gated ion channels. In particular, the orientation of membrane spanning for regions IV, V, VI, and VIII of the *trp* proteins could be the same as that of the S3, S4, S5, and S6 of the voltage-gated ion channels. Thus, the C termini of the *trp* proteins would be located at the cytoplasmic side of the membrane (Fig. 4b). Also, region VII of the *trp* proteins would be equivalent to the H5 region of the voltage-gated ion channels, which does not span across the membrane but enters the lipid bilayer partially and, in conjunction with S5 and S6, forms the pore through which ions cross the membrane. On the other hand, the first three hydrophobic regions of the *trp* proteins have no homology to the S1 and S2 transmembrane segments of the voltage-gated ion channels. All of these regions could span across the plasma membrane and thus bring the N-termini of the *trp* proteins to the extracellular side, assuming that *trp* is localized to the plasma membrane (Fig. 4b, middle). Consistent with this model, the presence of three putative glycosylation sites in the N-terminal domain of the *Htrp*-1 also suggests its extracellular localization. However, it is not clear whether *Htrp*-1 is glycosylated or if glycosylation is needed for its function since the other known *trp* proteins do not have consensus glycosylation sites. Alternatively, for *Drosophila trpl*, the third hydrophobic segment was not suggested to be transmembrane so that its N-terminus was localized at the cytoplasmic side as in the case of the voltage-gated ion channels [9] (Fig. 4b, lower). This is possible since a peptide sequence rich in hydrophobic residues may not always give rise to a transmembrane segment. An example of this kind was recently shown for the glutamate receptors, for which the original study of its hydropathy plot had suggested 4 transmembrane segments. However, recent reports from several groups have shown that the second segment is actually located on the cytoplasmic side without transversing the plasma membrane [22]. Therefore both models depicted in Fig. 4b (middle and lower) are possible for *trp* proteins. The true membrane topology of *trp* proteins needs to be investigated thoroughly through experimental approaches.

Nonetheless, the S4 segments of the voltage-dependent ion channels contain several positively charged amino acid residues which is thought to form the voltage sensors whereas the equivalent region V of the known *trp* proteins contains only one positive residue (Fig. 4a), suggesting that *trp* is unlikely to be gated by changes in membrane potentials.

Several lines of evidence have suggested that the *Drosophila trp* proteins are involved in the Ca^{2+} influx in the IP_3 mediated signaling pathways. In *Drosophila* photoreceptors, *trp* has been shown to form a light-sensitive Ca^{2+} permeant channel [20] and in *Sf*9 cells infected by baculovirus carrying cDNA for either

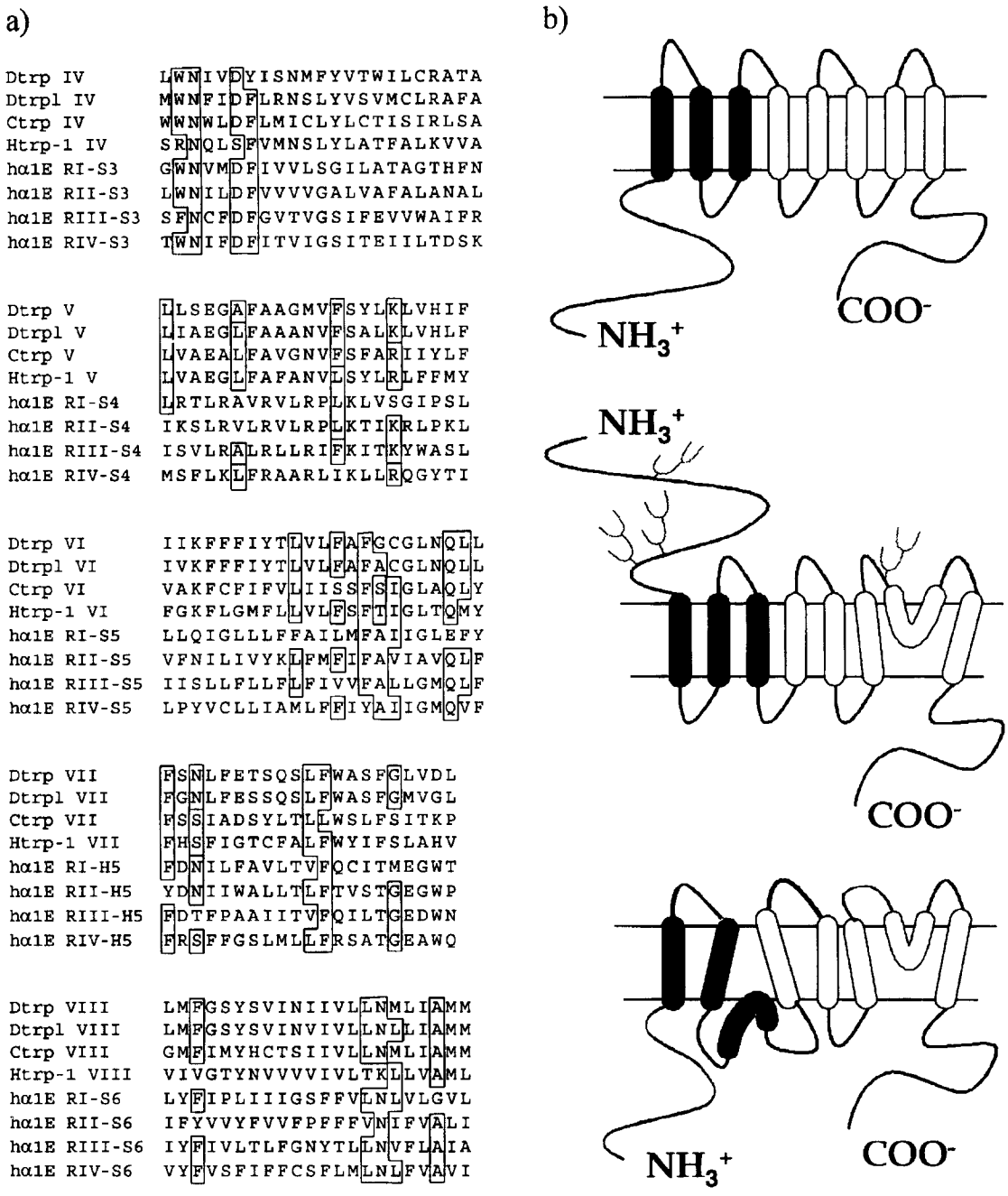

Fig. 4. (a) Comparison of hydrophobic regions IV to VIII of the *trp* proteins to the transmembrane segments S3 through S6 and the pore region (H5) of the four domains of α1E subunit of a human voltage-dependent Ca^{2+} channel [21]. The domains for the Ca^{2+} channel are indicated as D1 through D4. The abbreviations for *trp*'s are the same as in Fig. 1. (b) Possible membrane topologies for a *trp* protein. Upper: transmembrane organization of *Htrp*-1 based on the hydropathy plot. Middle: a possible topology based on the similarities between hydrophobic regions IV, V, VI, and VIII of *trp*'s and transmembrane segments S3 to S6 of voltage-gated ion channel. Region VII of the *trp* may form the pore. Region I, II, III of the *trp* form additional three transmembrane segments, bringing the N-terminus to the extracellular side. The tree-like structures show the putative glycosylation sites in *Htrp*-1. The lower panel illustrates the presumptive membrane topology of *Drosophila trpl* according to the description by Phillips et al. [9].

trp or *trpl*, the expressed protein was found to form Ca^{2+} permeant channels that functions in the IP_3/Ca^{2+} signaling pathway [10–13]. Therefore, it is reasonable to assume that the human *trp* may also play a role in the IP_3/Ca^{2+} signaling pathway. Unlike the two *Drosophila trp* proteins, which are expressed only in the insect's photoreceptor cells [7,9], the human *trp* homologue is expressed in most human tissues. Whether *Htrp*-1 forms a Ca^{2+} permeant channel that can be regulated by store-depletion or receptor activation still needs to be investigated. On the other hand, the levels of the *Htrp*-1 transcript in most non-excitable tissues are relatively low, especially in liver. Since Ca^{2+} influx occurs most significantly in non-excitable cells, other types of *trp* homologues may be present in these cells to fulfill the influx function. The cloning of the *Htrp*-1

provides the sequence information for searching for additional members of the *trp* protein family in mammalian tissue.

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