BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 279–283 (1997)
ARTICLE NO. RC977458

Differential Expression of Mammalian *TRP* Homologues across Tissues and Cell Lines

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Received September 8, 1997

Mammalian homologues of the *Drosophila trp* gene have been invoked as the structural basis for the currents associated with capacitative Ca^{2+} entry (CCE) in many cell types. *Trp* homologues are members of a large protein family that may associate as channel subunits providing an explanation for the functional diversity of store-operated channels observed in these cells. However, there is little information as to which of these genes are co-expressed at the cellular level. We have examined the tissue specific expression of five mammalian *trp* genes and determined which are co-expressed in five different cell lines. The results show tissue- and cell-specific co-expression of multiple *trp* forms. This implies that the subunit composition of a particular CCE channel may vary depending on the cell type.

Capacitative Ca^{2+} entry is loosely defined as the influx of Ca^{2+} from the extracellular space following inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3)-induced mobilization of internal Ca^{2+} stores [1]. This process, otherwise known as store-operated Ca^{2+} entry, has been demonstrated in a variety of cell types [2-9] although the ionic currents associated with Ca^{2+} entry in the various cells seem to differ in unitary conductance and ion selectivity [10-12]. The Ca^{2+} release-activated Ca^{2+} current, or I_{CRAC}, described in mast cells [2] and T lymphocytes [4,5] is the best characterized of the CCE currents, and I_{CRAC}-type channels appear to be present in rat basophilic leukemia cells (RBL) [2,3], hepatocytes [13,14] and vascular endothelial cells [8]. I_{CRAC} is characterized by an unusually low conductance (sub-picosiemen) and a very high selectivity for Ca^{2+} [2,5]. Store-depletion-sensitive, non-selective cation currents and channels with higher conductances than I_{CRAC} have also been reported [10,11].

The *trp* gene of *Drosophila* [15,16] and its homologue, *trp-like* [*trpl*; 17], were suggested to encode the functional equivalents of mammalian CCE channels [18]. Indeed, functional expression of *trp* in Sf9 cells [19] and 293T cells [20] causes the appearance of novel membrane currents that show modest selectivity for Ca^{2+} and are sensitive to store depletion. In contrast, *trpl* encodes a non-selective cation channel that is constitutively active when heterologously expressed [20,21]. To date, six mammalian *trp* homologues have been isolated, three of which have been reported in their full length[1] (*trp-1, trp-3 and trp-4*) and expressed in heterologous systems [22-25]. Based on the available sequences covering the highly conserved S5-S6 regions, these genes were grouped into four subfamilies where *trp*(*s*) *3* and *6* belong to one subfamily and *trp*(*s*) *4* and *5* to another [24,26]. The most compelling evidence to date suggesting that these *trp* homologues are involved in CCE are the studies of Zhu et al [26], which showed that expression of antisense *trp* sequences abolished capacitative Ca^{2+} entry leading to speculation that the six *trp* homologues are subunits of the CCE channels. Nevertheless, the subunit composition of a particular CCE channel and definition of subfamily-specific rules which might govern subunit assembly (as for voltage-gated K+ channels [27]) remain to be determined. In the present study, we evaluated the tissue specific expression of 5 mammalian *trp* homologues and determined which transcripts are co-expressed in five different cell lines. The results suggest that multiple *trp* homologues are expressed at the tissue and cellular levels, but no obvious patterns, suggestive of specific channel subunit composition, has emerged.

MATERIALS AND METHODS

Tissues and cell lines. Tissues were obtained from 4-week old Sprague-Dawley rats, frozen in liquid nitrogen, and stored at −80°C until use. Bovine aortic endothelial cells (BAECs) and human fibroblasts (SK 45) were cultured as previously described [28,29]. Jurkat

[1] The nomenclature of Zhu et al [26] was followed in this paper. Hence *trpC1* [34], *trpC3* [20,34], and bCCE/*trp-R* [22,35] are *trp-1*, *trp-3* and *trp-4*, respectively.

0006-291X/97 $25.00

TABLE 1A

Sequences of Primers Used for RT-PCR Expression Profiling

Gene	Acc. No.	Orientation	Sequence (5′ to 3′)	Product size
mtrp-1	U40980	Forward	CAAGATTTTGGGAAATTTCTGG	372
		Reverse	TTTATCCTCATGATTTGCTAT	
htrp-3	Y13758	Forward	TGACTTCCGTTGTGCTCAAATATG	317
		Reverse	CCTTCTGAAGCCTTCTCCTTCTGC	
mtrp-4	X90697	Forward	TCTGCAGATATCTCTGGGAAGGATGC	415
		Reverse	AAGCTTTGTTCGAGCAAATTTCCATTC	
mtrp-5	U40984	Forward	ATCTACTGCCTAGTACTACTGGCT	340
		Reverse	CAGCATGATCGGCAATGAGCTG	
mtrp-6	U49069	Forward	AAAGATATCTTCAAATTCATGGTC	327
		Reverse	CACGTCCGCATCATCCTCAATTTC	
cyc	M19533	Forward	GCAGACATGGTCAACCCCACCG	504
		Reverse	GAAATTAGAGTTGTCCACAGTCGG	

cells (human acute leukemic T cells) were grown in RPMI 1640 plus 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/neomycin (PSN) and 1% L-glutamine (LG). Rat basophilic granulocytes (RBL) were grown in minimal essential medium (MEM) plus 10% FBS and 1% PSN. Human embryonic kidney cells (HEK 293) were cultured in MEM plus 10% FBS, 1% PSN and 1% LG.

Total RNA extraction, first strand cDNA synthesis, and PCR amplification. Total RNA was extracted by the method of Chomcyznski and Sachii [30]. First strand cDNA was generated using MMLV reverse transcriptase and oligo-dT and random primers as previously described [31]. PCR was performed with Taq polymerase for 25 or 35 cycles using a Perkin-Elmer DNA Thermal Cycler 480. Primers used for amplification of mammalian *trp* gene fragments (**Table 1A**) were based on either mouse or human *trp* sequences [26,34]. The thermocycler was programmed to give an initial cycle consisting of 94°C denaturation for 5 min, 55 or 57°C annealing for 1 minute, and 72°C extension for 1 min followed by 24 or 34 cycles of 94°C, 55 or 57°C, and 72°C for 1 minute each. A final extension at 72°C for 5 minutes was included only for samples given 35 cycles of amplification. A "no-template control" was performed alongside all experimental samples. In all manipulations, UV-treated tubes and sterile filtered pipette tips were used to prevent tissue-to-tissue and aerosol contaminations. Separate pipettors and areas were also used for pre- and post-PCR.

Sequence-specific hybridization and DNA sequencing. To verify the identity of the PCR products, Southern blots were hybridized overnight at 50 to 55°C with a ^{32}P-5′end-labeled synthetic oligonucleotide probe (**Table 1B**), that recognizes the sequence flanked by the two primers used for PCR amplification of each *trp* transcript. The results shown are representative of 3 to 5 RNA extracts from each tissue or cell line. Where indicated, PCR products were subcloned into pGem T-Easy (Promega) and sequenced by the dideoxy chain termination method using Sequenase Version 2.0.

RESULTS

Tissue distributions of mammalian trp genes. The expression of five mammalian *trp* homologues was examined in various tissues from adult rat. Using RT-PCR, stopped during the pre-determined exponential phase of amplification, the relative abundance of a particular *trp* transcript was compared across tissues. Amplification for 35 cycles, which is beyond the exponential phase, was initially carried out to detect low levels of expression which may otherwise be missed by Northern blot hybridization or RNAse protection assay. The expression profile of cyclophilin was included as an endogenous internal control for loading and efficiency of the RT and PCR steps. The primers chosen fall in a region of mammalian *trp* genes known to cross a sizable intron [26], thus eliminating contribution from genomic DNA amplification. The use of radiolabeled synthetic oligonucleotides internal to the primers used for PCR, as probes for Southern blot hybridization, further excluded similar-sized non-target transcripts or primer-generated artifacts.

All mammalian *trp* homologues examined are most abundantly expressed in the brain (cerebrum or cerebellum) and barely detectable in the liver (**Fig. 1**). *Trp-1* mRNA is most abundant in the cerebrum, heart, testis, and nodose ganglia, whereas moderate levels of

TABLE 1B

Sequences of Oligonucleotides Used as 5′ End-Labeled Probes

Gene	Acc. No.	Orientation	Sequences (5′ to 3′)
mtrp-1	U40980	sense	GAACAGCAAAGCAATGACAC
htrp-3	Y13758	anti-sense	GGAACTAGACTGAAAGGTGGAGG
mtrp-4	X90697	anti-sense	TATATCCGCATGGTCAGCAATAAG
mtrp-5	U40984	sense	ATGAACCTAACAACTGCAAGG
mtrp-6	U49069	sense	GTCTTTCTGAAGTGAAGTCAG
cyc	M19533	sense	CGACATCACGGCTGATGGCGAGCCC

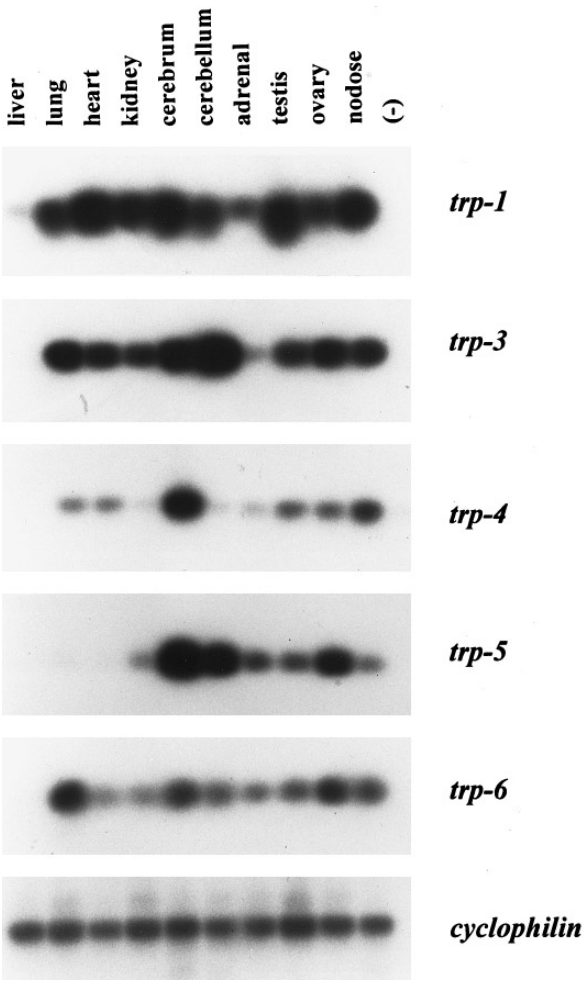

FIG. 1. Expression of mammalian *trp* homologues in adult rat tissues. The indicated genes were amplified by RT-PCR within their pre-determined exponential phase of amplification, blotted and probed with 5′ end-labeled synthetic oligonucleotides internal to the primers used for PCR. The column labeled (−) reflects PCR reactions performed without template.

expression are observed in the lung, kidney, cerebellum and ovary. Much weaker expression is detected in adrenal gland and even lower levels are detected in the liver. Our results are similar to those of Zhu et al [33] and Wes et al [34] who found high levels of *trp-1* mRNA in the adult human brain, heart, testis and ovary using Northern blot analyses. For *trp-3*, expression is highest in the cerebellum and moderate levels of expression are observed in all other tissues except the adrenal gland, which shows much weaker expression, and the liver, which has barely detectable levels of *trp-3* transcripts. Zhu et al [26] also observed a preponderance of *trp-3* mRNA in the human brain and weaker expression in most other tissues they examined; both *trp-1* and *trp-3* were not detected in adult human liver by Northern blot hybridization [26,33,34].

Trp-4 is predominant in the cerebrum and moderate amounts are also present in the nodose ganglia, lungs, heart, testis and ovary. Weaker bands are observed in the kidney, adrenal gland and cerebellum, whereas the liver has barely detectable levels of the transcript even after 35 cycles of amplification. Funayama and coworkers [35] showed high levels of *trp-4* (which they called *trp-R* but appears to be a splice variant of *trp-4*) in the brain of late embryonic and postnatal rats. The limited sensitivity of Northern blot analysis, however, failed to detect lower levels of expression in all other tissues they examined leading them to suggest that *trp-4* is brain-specific.

Trp-5 showed the most variable pattern of expression among mammalian *trp* (*s*). *Trp-5* is most abundant in the cerebrum followed by the cerebellum and the ovary. Moderate amounts of the transcript are also detected in the adrenal gland, testis, kidney and nodose ganglia. Much weaker expression is observed in liver, lungs and heart. *Trp-6* expression is highest in the lungs, cerebrum and ovary. Moderate levels of expression are observed in all other tissues except the liver which shows barely detectable levels of the transcript even after 35 cycles of amplification. Interestingly, *trp-6* transcripts are very easily amplified in great amounts from liver total RNA of neonates (postnatal day 3; data not shown) suggesting that this gene is developmentally regulated.

Co-expression of multiple trp genes in specific cell lines. We determined which mammalian *trp* homologues are expressed in 5 different cell lines, three of which have been shown to exhibit I_{CRAC}. Amplification was allowed to proceed for 35 cycles to increase the chances of detecting low levels of expression. Expression of multiple *trp* genes is observed in all cell lines examined (**Fig. 2**). In BAECs, all five *trp* genes were detected. Based on repeated experiments involving amplification from different RNA extracts at varying cycle numbers and evaluation of transcript levels on agarose gels by ethidium bromide staining, it is apparent that *trp-1* and *trp-4* are the predominant transcripts in BAECs and *trp-6* is very difficult to amplify. In addition, four (*trp-1, -3, -4 and -5*) of the five *trp* genes from BAECs were also verified by sequencing. The predicted amino acid sequences were identical to those of the corresponding genes in mouse and human, although marked differences in codon usage were observed, confirming that they are bovine in origin and were not the result of contamination.

In Jurkat cells, *trp-6* is the predominant transcript followed by *trp-3*, although *trp*(*s*) *1* and *5* are also detected; the *trp-4* transcript is not detectable in Jurkat cells even after 35 cycles of amplification. In RBL cells, *trp-1* is much more easily amplified than *trp-5*, and *trp-4* and *trp-6* are very weakly detected; *trp-3* is not detectable in RBL cells. In human fibroblasts (SK 45), the profile is very similar to that seen for BAEC; *trp*(*s*)

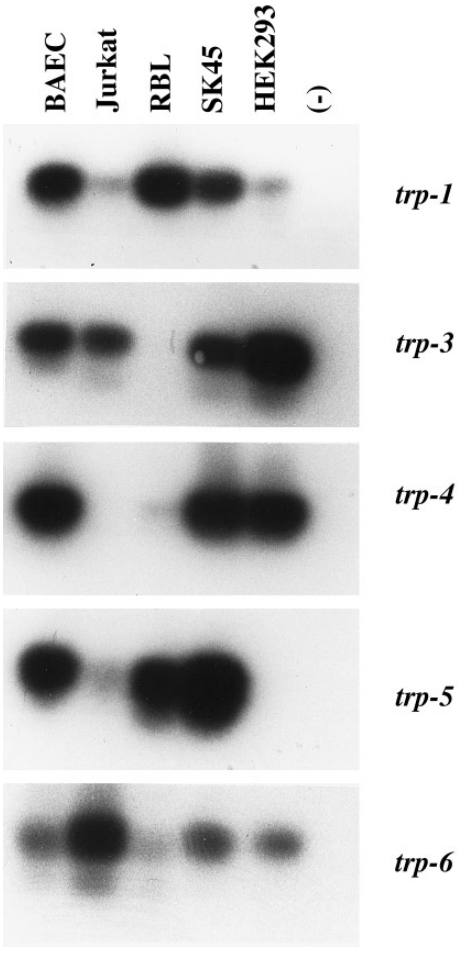

FIG. 2. Expression of mammalian *trp* homologues in various cell lines. Experimental procedures as for Fig. 1 with the exception that amplification was allowed to proceed for 35 cycles to detect lower levels of expression in each cell line.

1, 3, 4 and *5* are quite highly expressed and *trp-6* is also present in moderate amounts. *Trp-3* is very highly expressed in HEK 293 cells; *trp*(*s*) *1, 4* and *6* are also present, but *trp*-5 was not detected. Although no data was shown, Sakura & Ashcroft [36] mentioned that they also detected *trp*(*s*) *1, 3, 4* and *6* and failed to detect *trp-5* transcripts from HEK 293 poly A+ RNA.

DISCUSSION

The data presented here show for the first time a distinct pattern of tissue distribution for mammalian *trp* genes. All are most abundantly expressed in the brain and are barely detectable in the liver, observations difficult to reconcile with the fact that CCE is predominantly observed in non-excitable cells and that hepatocytes represent one of the best models for capacitative Ca^{2+} entry. Our results also show that some or all of these genes may be co-expressed in the same cell type, although at varying levels. More importantly, three cell lines known to exhibit I_{CRAC} do not have the same pattern of expression for these genes and the predominant transcript seems to be different in any one cell type. Most notably, Jurkat cells predominantly express *trp-3* and *trp-6* and lack *trp-4*, whereas RBL cells predominantly express *trp-1* and *trp-5* and lack *trp-3* despite the fact that these cell types exhibit remarkably similar I_{CRAC} currents [12]. Our inability to amplify certain *trp* transcripts from some cell lines is not a function of primer design or secondary structure, as the same primer pairs very easily amplified these same target transcripts from other tissues and cell lines as shown in Figs. 1 and 2, suggesting that the results observed are indeed indicative of relative abundance.

The significance of the unexpected profiles described above is difficult to assess since mRNA levels do not always equate with protein levels (owing to different modes of regulation) and because different cells may require different amounts of the protein products encoded by these genes. In this regard, the low level of expression of *trp* genes in the liver may simply reflect cell type-specific requirements for these proteins. Moreover, whereas some of these transcripts may simply be the result of leaky or ectopic transcription [37], some are abundantly co-expressed at comparable levels suggesting that co-expression may be functionally relevant. Whether the coexpressed proteins encoded by these genes form part of the same channel or are subunits of different channels found in the same cell type remains an open question. Another issue is whether these channel subunits are functionally similar or functionally distinct. Is CCE, in fact, subserved by different channel compositions in different cell types? If these subunits are functionally similar, they need not be co-expressed in the same cell type unless functional redundancy is allowed. If, however, they are functionally distinct from each other, then it is more likely that they should be expressed in the same cells. A third possibility is that these subunits are isofunctional and represent the common subunit of distinct channels within the same cell, but that other subunits and accessory proteins yet unidentified are needed to bring about particular channel characteristics. It must be noted however, that potential subunits of CCE channels may not be limited to these six genes. Splice variants involving the coding region have already been identified for both *trp-1* and *trp-4*, and it is likely that this phenomenon is widespread among mammalian *trp*(*s*). It will be important to determine whether alternative splicing within the *trp* family has functional significance or has simply evolved as a means to facilitate differential expression of functionally similar subunits in different cells.

In summary, we have shown that mammalian *trp* genes are ubiquitously expressed, although at varying

levels. We have also shown that multiple mammalian *trp* genes are co-expressed in any one particular cell line and that these cells do not have the same pattern of expression for these genes. The former implies that these genes may encode subunits of distinct channels in one given cell. The latter suggests that the subunit composition of a particular CCE channel, such as I_{CRAC}, may be different depending on the cell type. Given the very low expression of mammalian *trp* genes in liver compared to other tissues, the possibility that there are other genes coding for CCE channel subunits, which are not necessarily similar to *trp* family members in sequence, should also be considered.

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