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A novel capacitative calcium entry channel expressed in excitable cells

Stephan Philipp[1], Joerg Hambrecht, Leonid Braslavski, Gregor Schroth, Marc Freichel, Manabu Murakami, Adolfo Cavalié and Veit Flockerzi

Institut für Pharmakologie und Toxikologie der Universität des Saarlandes, D-66421 Homburg/Saar, Germany

[1]Corresponding author
e-mail: stephan.philipp@med-rz.uni-sb.de

In addition to voltage-gated calcium influx, capacitative calcium entry (CCE) represents a major pathway for calcium entry into the cell. Here we report the structure, expression and functional properties of a novel CCE channel, TRP5. This channel is a member of a new subfamily of mammalian homologues of the *Drosophila* transient receptor potential (TRP) protein, now comprising TRP5 (also CCE2) and the structurally related CCE1 (also TRP4). Like TRP4, TRP5 forms ion channels mainly permeable for Ca^{2+} which are not active under resting conditions but can be activated by manoeuvres known to deplete intracellular calcium stores. Accordingly, dialysis of TRP5-expressing cells with inositol-(1,4,5)-trisphosphate evokes inward rectifying currents which reversed polarity at potentials more positive than +30 mV. Ca^{2+} store depletion with thapsigargin induced TRP5-mediated calcium entry dependent on the concentration of extracellular calcium, as seen by dual wavelength fura-2 fluorescence ratio measurements. TRP5 transcripts are expressed almost exclusively in brain, where they are present in mitral cells of the olfactory bulb, in lateral cerebellar nuclei and, together with TRP4 transcripts, in CA1 pyramidal neurons of the hippocampus, indicating the presence of CCE channels in excitable cells and their participation in neuronal calcium homeostasis.

Keywords: capacitative calcium entry/$InsP_3$/store depletion/store-operated calcium channel/TRP5

Introduction

Activation of the inositol-(1,4,5)-trisphosphate ($InsP_3$) signalling cascade by neurotransmitters, hormones and growth factors leads to the formation of $InsP_3$ which binds to $InsP_3$ receptors and thereby initiates the release of Ca^{2+} from intracellular stores. Depletion of the stores is followed by the influx of Ca^{2+} into the cell through store-operated Ca^{2+}-selective plasma membrane channels, a mechanism which has been described as capacitative calcium entry (CCE; Putney, 1986). Ion currents associated with CCE have been studied extensively in non-excitable tissues (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Lückhoff and Clapham, 1994). However, neurons, like virtually all cells, have $InsP_3$- and ryanodine-sensitive Ca^{2+} stores, and it has been envisaged for some time that in excitable cells Ca^{2+} release from intracellular stores and Ca^{2+} influx into the cell may also be coupled (Stühmer and Parekh, 1993; Gomez *et al*., 1995; Garaschuk *et al*., 1997). Store-operated channels that have been described so far differ in their biophysical properties, including ion selectivity and single channel conductance, but their molecular nature has not been clearly identified.

Two photoreceptor cell-specific gene products have been isolated from *Drosophila melanogaster*, which have been implicated to function as Ca^{2+} entry channels. The transient receptor potential (*trp*) gene product is a protein of 1275 amino acids with multiple transmembrane domains (Montell and Rubin, 1989). Heterologous expression of *trp* cDNA in eukaryotic cells led to the formation of ion channels which could be activated by the depletion of intracellular Ca^{2+} stores (Petersen *et al*., 1995) and which were primarily permeable to Ca^{2+} (Vaca *et al*., 1994). The transient receptor potential-like (*trpl*) gene product is 39% identical to TRP (Phillips *et al*., 1992), but *in vitro* studies revealed that TRPL is a non-selective cation channel which is not store operated but constitutively active (Hu *et al*., 1994; Hu and Schilling, 1995; Zimmer *et al*., 1997). Both proteins may assemble into heteromultimeric complexes leading to ion channels with different ion selectivity compared with those of homomultimeric channels (Gillo *et al*., 1996; Xu *et al*., 1997). It has also been proposed that TRP–TRPL heteromultimeric complexes are associated with additional proteins such as phospholipase C, protein kinase C and the PDZ-domain protein inactivation-no-afterpotential D (INAD) (Huber *et al*., 1996; Chevesich *et al*., 1997; Tsunoda *et al*., 1997).

Recently, the cDNAs of mammalian homologues of the dipterian TRP/TRPL proteins have been cloned. One group of cDNAs including *htrp1*, *htrp3* and *mtrp6* encodes Ca^{2+}-permeable but non-selective cation channels when expressed in eukaryotic cells (Zitt *et al*., 1996; Boulay *et al*., 1997; Zhu *et al*., 1998). The recombinant channels hTRP1 and hTRP3 are active under resting conditions (Sinkins and Schilling, 1997; Hurst *et al*., 1998), which may reflect the lack of auxiliary subunits that may regulate channel activity. In addition, hTRP3 and mTRP6 appear to be insensitive to Ca^{2+} store depletion (Boulay *et al*., 1997; Zitt *et al*., 1997).

A bovine homologue of TRP/TRPL, bCCE (now bCCE1, also bTRP4) represents the first member of another group of cDNAs, which—when expressed in human embryonic kidney (HEK) cells (Philipp *et al*., 1996) or Chinese hamster ovary (CHO) cells (Warnat *et al*., 1998)—confers a store-operated, highly Ca^{2+}-permeable ion channel to these cells. Additional evidence that TRP4 underlies CCE came from recent studies in which cDNA fragments of the murine TRP4 in antisense

orientation were transfected into mouse L cells resulting in an inhibition of the endogenous CCE (Birnbaumer *et al*., 1996).

We have now isolated cDNAs from rabbit and mouse brain encoding a protein TRP5 which shows significant homology to the dipterian TRP (41% amino acid identity) and bovine TRP4 (69% amino acid identity). Expression of TRP5 in HEK cells was sufficient to induce the formation of a plasma membrane channel which was mainly permeable for Ca^{2+} and was activated by $InsP_3$ or thapsigargin via depletion of intracellular Ca^{2+} stores. Accordingly, TRP5 together with TRP4 are the first members of the subfamily of CCE channels structurally related to TRP/TRPL from *Drosophila*. Due to the expression pattern of TRP5 transcripts in brain, the TRP5 channel may be responsible for CCE in certain neurons.

Results

Primary structure of rabbit and mouse TRP5

We amplified a 133 bp cDNA fragment C (Figure 1A) from rabbit brain using degenerate oligonucleotides derived from TRP and TRPL. By screening brain cDNA libraries, the full-length cDNA sequence of rabbit TRP5 (rTRP5) was obtained. The translation initiation site was assigned to the first ATG triplet that appears downstream of a nonsense codon found in-frame. The deduced amino acid sequence of rTRP5 is composed of 974 residues, with a calculated molecular mass of 111 533 Da (Figure 1B). Hydropathy analysis reveals a hydrophobic core in the rTRP5 protein with six peaks likely to represent membrane-spanning helices (Figure 1C; S1, S2, S3, S4, S5 and S6) and a putative pore region between S5 and S6. The hydrophobic core is flanked by long presumptive cytoplasmic domains at the N- and C-termini. A similar topology has been proposed for TRP, TRPL and their known mammalian homologues including bTRP4. Consistent with this topology, two potential cAMP-dependent phosphorylation sites within rTRP5 (Ser122 and Thr167) are located in the cytoplasm. As shown in Figure 1B, rTRP5 shares 68.5% sequence identity with the bTRP4 protein. Their N-termini (residues 1–329 in rTRP5) and the hydrophobic cores (residues 330–624 in rTRP5) share considerable amino acid identity of 80.2 and 84.9%, respectively, whereas the C-terminal regions of rTRP5 and bTRP4 reveal a lower level of identity (42.4%). In contrast, amino acid sequence comparison of rTRP5 reveals 34.5 (bTRP2), 40.6 (dTRP), 41.1 (dTRPL), 41.7 (mTRP6), 42.9 (hTRP3) and 47.3% (hTRP1) identity with TRP and its homologues.

Using degenerate oligonucleotides corresponding to the N- (amino acids 1–8) and C-termini (amino acids 959–965) of rTRP5, the mouse TRP5 (mTRP5) cDNA was

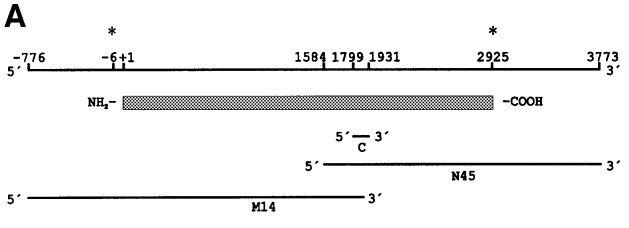

Fig. 1. Primary structure of TRP5. (**A**) Cloning strategy: lines indicate cDNAs with a translation start site at position +1, a bar represents the corresponding protein. Stop codons in-frame are indicated by asterisks. (**B**) The deduced amino acid sequence of TRP5 from rabbit brain (rTRP5) is shown in alignment with the sequence from mouse brain (mTRP5) and with the bovine TRP4 sequence (Philipp *et al*., 1996). Amino acid residues are numbered on the right; numbering of mTRP5 is in accordance with that of rTRP5. Residues within mTRP5 and bTRP4 identical to rTRP5 are indicated by blanks, gaps by dashes, and the location of stop codons by asterisks. Limits of the mTRP5 sequence obtained are denoted by < >. The putative transmembrane segments (S1–S6) and the putative pore region are indicated. (**C**) Hydropathy profile (Kyte and Doolittle, 1982) of rTRP5: transmembrane segments S1–S6 were defined as regions with a hydropathy index ⩾1.5 using a window of 19 amino acids.

amplified by RT–PCR from mouse brain mRNA as template. The mTRP5 cDNA covers a sequence of 369 bp recently amplified from mouse brain (Zhu *et al.*, 1996). The mTRP5 protein shares 97.5% amino acid sequence identity with rTRP5, with few exchanges, mainly within the C-terminus, indicating a high conservation throughout evolution.

Expression of TRP5 in excitable cells of the brain—co-expression with TRP4

By Northern analysis, we found that TRP5 mRNA is expressed predominantly in the brain. The rTRP5 probe hybridized with transcripts of 4.2, 8.0 and 10.5 kb in rabbit brain and to a much lower degree in kidney (Figure 2A). No TRP5 transcripts were detected in liver, ureter, ovary, lung, aorta, spleen and thymus, or in adrenal gland and testis, where TRP4 transcripts primarily are expressed (Philipp *et al*., 1996). The 4.2 kb mRNA corresponds to the size of the rTRP5 cDNA (4549 bp) cloned from rabbit brain (Figure 1A). The longer transcripts may result from alternative mRNA processing. In fact, cDNA clones, which differ in their 5′- and 3′-untranslated sequences, were isolated from the cDNA libraries employed. In human brain, the rTRP5 probe hybridized with transcripts of 10.5 kb (Figure 2B), indicating a different pattern of hybridization signals as compared with rabbit mRNA, presumably due to species-specific TRP5 gene structure and mRNA processing. The TRP5 mRNA in human brain is present in the cerebellum and the occipital pole, and at a lower level in the medulla and the frontal lobe. To localize the transcripts in cerebellum, we used *in situ* hybridization histochemistry. As shown in Figure 2C, mTRP5 mRNA is present in neurons of the lateral cerebellar nucleus. In addition, TRP5 transcripts were detected in the entorhinal cortex (not shown). Thus, the main expression of TRP5 seems to occur in excitable cells of the brain, in contrast to the expression of other mammalian TRP-related proteins which are expressed predominantly, but not exclusively, in non-neuronal tissues (Wes *et al*., 1995; Philipp *et al*., 1996; Zhu *et al*., 1996; Boulay *et al*., 1997; Wissenbach *et al*., 1998).

At present, we do not know whether CCE channels are heteromultimeric in nature and whether TRP5 co-assembles with other TRP homologues, e.g. TRP4. In the mouse brain, we found TRP5 transcripts throughout the Ammon's horn (CA) of the hippocampus (Figure 3A, left panel). Using TRP4 cRNA as probe, a very similar hybridization pattern was obtained (Figure 3A, right panel), indicating that both channels might be expressed in the same cells. In fact, TRP5 and TRP4 transcripts appear to be co-expressed in CA1 hippocampal pyramidal neurons (Figure 3B). TRP5 and TRP4 mRNAs were also detected in cells within the olfactory bulb (Figure 3C and D). TRP5 transcripts seem to be restricted to the mitral cell layer and the glomerular layer (Figure 3C and D, left panel), whereas TRP4 transcripts are expressed in the internal granular layer (Figure 3C, right panel), but appear not to be present in mitral cells (Figure 3D, right panel). Apparently, depending on the tissue and the cell type, co-expression of TRP5 and TRP4 transcripts may occur.

The TRP5 protein is a CCE channel

To examine the functional properties of TRP5, HEK cells were transiently transfected with the cDNA of rTRP5. We used a dicistronic expression vector (see Materials and methods) which contains the rTRP5 cDNA upstream of an internal ribosome entry site (IRES) followed by the cDNA of the green fluorescent protein (GFP). The IRES sequence allows the simultaneous translation of TRP5 and GFP from one transcript. Thus, transfected cells can be detected unequivocally by the development of green fluorescence. In order to empty intracellular Ca^{2+} stores, the transfected cells were dialysed with 10 μM $InsP_3$

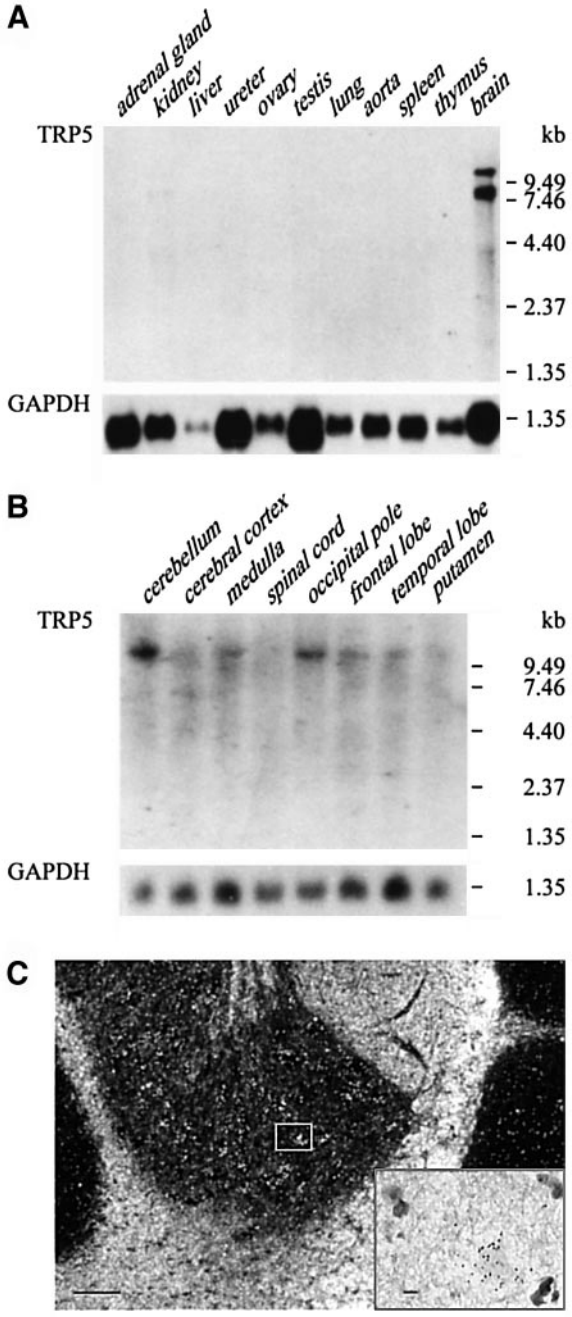

Fig. 2. TRP5 is expressed predominatly in brain. Northern blot analysis of rTRP5 expression in rabbit tissues (**A**) and in human brain (**B**). Lower panels show signals after hybridization of human GAPDH cDNA to the same filters. (**C**) *In situ* hybridization of mTRP5 antisense cRNA to a horizontal section of the mouse lateral cerebellar nucleus (dark-field microscopy). A white rectangle indicates the location of a magnification shown as an inset (bright-field microscopy). Bars represent 100 and 10 μm (inset).

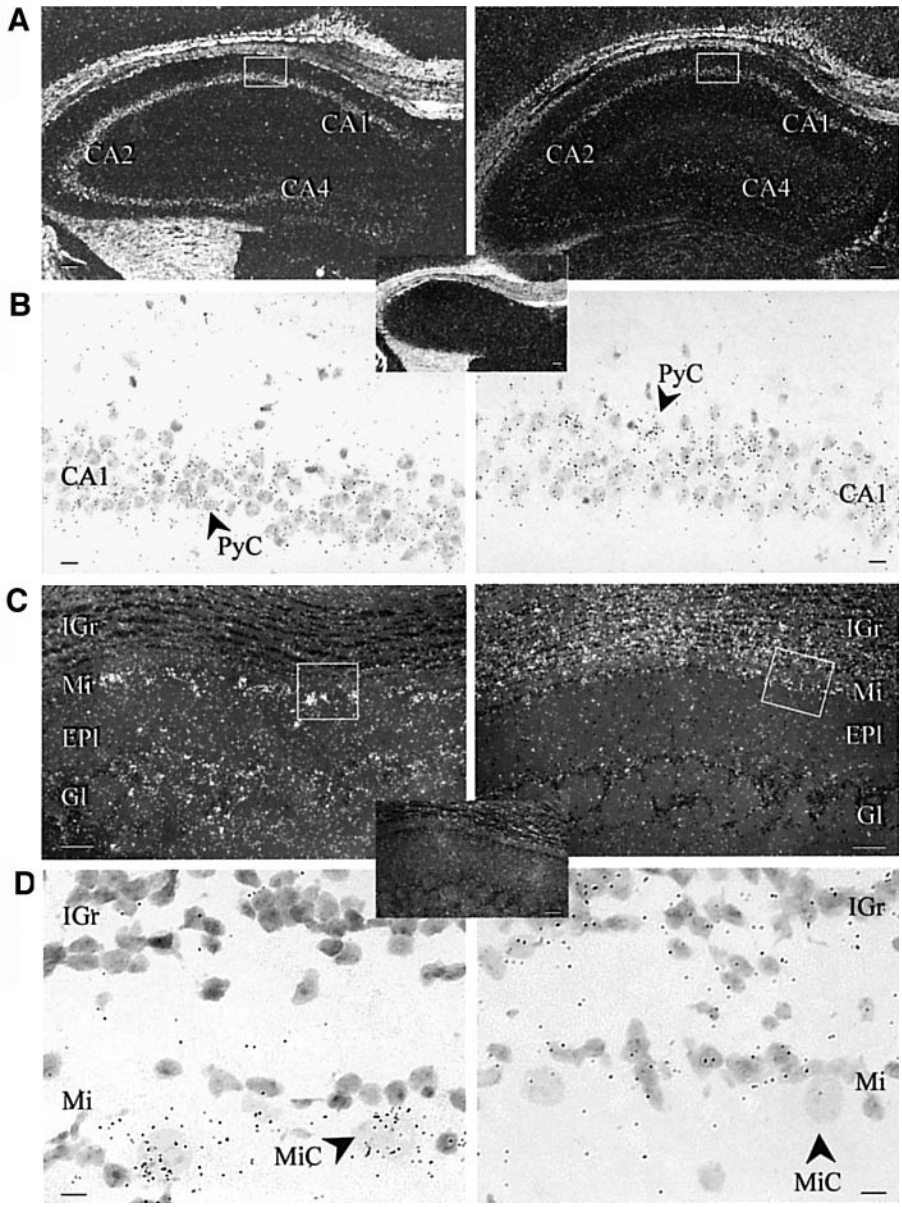

Fig. 3. TRP5 and TRP4 are co-expressed in hippocampal cells but not in the olfactory bulb. *In situ* hybridization of mTRP5 (left panels) and mTRP4 (right panels) antisense cRNA to frontal sections through the hippocampus (**A** and **B**) and the olfactory bulb (**C** and **D**) of a mouse brain. Insets show controls using sense cRNA probes. White rectangles indicate the locations of magnifications shown in (B) and (D) (bright-field microscopy). Bars represent 100 μm (A, C and insets, dark-field microscopy) and 10 μm (B and D). CA, Ammon's horn; PyC, CA1 pyramidal cells; IGr, internal granular layer; Mi, mitral cell layer; EPl, external plexiform layer; Gl, glomerular layer; MiC, mitral cell. Note the expression signals in pyramidal cells and their presence or absence in mitral cells (arrowheads).

via a patch–clamp pipette. Additionally, the dialysate contained 10 mM EGTA to buffer the intracellular Ca^{2+} below 50 nM. Whole-cell currents were elicited with voltage-clamp ramps from –100 to +60 mV every 5 s (holding potential 0 mV) in the presence of 10 mM external Ca^{2+}. As the dialysis of the cells with $InsP_3$ progressed, a clear increase of inward currents was observed (Figure 4A). The inward currents develop 200 s after the onset of $InsP_3$ dialysis whereas the outward current remained stable throughout the experiment (Figure 4B). Analysis of leak-subtracted currents (Figure 4C) showed that TRP5 mediated inwardly rectifying currents. As expected for the dicistronic expression of TRP5 and GFP, similar inwardly rectifying currents were detected in eight out of nine green fluorescent cells. On average, the current density of TRP5-transfected cells was 6.71 ± 2.81 pA/pF (n = 8) at –80 mV. As previously reported (Philipp *et al*., 1996), $InsP_3$ also activated inward currents in non-transfected cells. The density of leak-subtracted inward currents was 0.57 ± 0.21 pA/pF (n = 15) at –80 mV in non-transfected HEK cells after dialysis with 10 μM $InsP_3$. Similar results were also obtained in HEK cells that were mock transfected with the same dicistronic vector containing lacZ instead of TRP5 (0.41 ± 0.13 pA/pF at –80 mV, n = 3). Thus, expression of TRP5 in HEK cells increased the amplitude of inward currents activated by $InsP_3$ ~11-fold.

The results shown in Figure 5 demonstrated that recombinant TRP5 currents display the typical features of currents associated with CCE, i.e. strong inward rectifica-

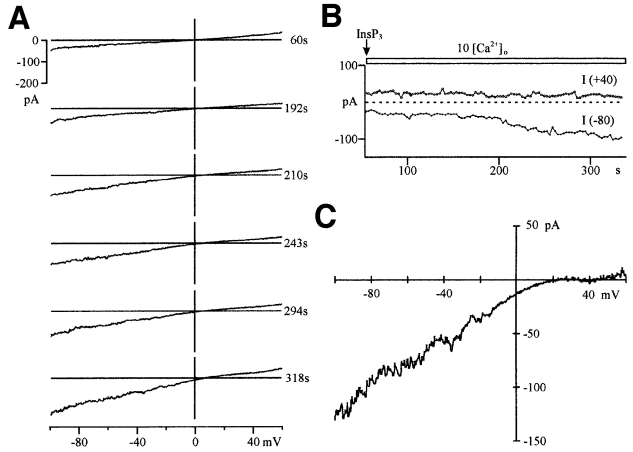

Fig. 4. Inward rectifying currents of TRP5-expressing HEK cells after store depletion. (**A**) Representative whole-cell currents in TRP5-transfected HEK cells during the dialysis with 10 μM $InsP_3$ in the presence of 10 mM extracellular Ca^{2+}, obtained at the times indicated and displayed without subtraction of leak currents. (**B**) Time course of current amplitudes at –80 mV and +40 mV. (**C**) Leak-subtracted current–voltage relationship of a TRP5-expressing cell 318 s after $InsP_3$ dialysis.

tion and a reversal potential above +30 mV. Additionally, these results indicate that recombinant TRP5 channels are probably Ca^{2+} selective like those detected in TRP4-transfected cells (Philipp *et al*., 1996; Warnat *et al*., 1998). Therefore, it can be expected that the CCE of cells expressing TRP5 is highly sensitive to changes in the extracellular Ca^{2+} concentration. We tested this idea by comparing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) dependence of CCE in control and TRP5-transfected cells. For these experiments, we established a HEK cell line, HekCCE2, that stably expresses the rTRP5 cDNA. As illustrated in Figure 5A, Northern analysis revealed the expression of 4.1 and 5.3 kb transcripts in HekCCE2 cells whereas no hybridization signals were detected in non-transfected HEK control cells. The length of the 5.3 kb mRNA in the HekCCE2 cells corresponds to the expected size for transcription of TRP5 in the dicistronic expression vector, whereas the 4.1 kb mRNA may arise from alternate RNA processing or cryptic transcription initiation (Kaufman *et al*., 1991).

Measurements of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) were conducted in steady state and during transient changes in the $[Ca^{2+}]_o$ below physiological levels to detect a possible Ca^{2+}-selective entry. In cells that were equilibrated in a solution containing 500 μM $[Ca^{2+}]_o$, the steady-state $[Ca^{2+}]_i$ was 107.7 ± 22.9 nM (n = 14), independent of TRP5 expression (Figure 5B). As has been reported for various cell types (e.g. Montero *et al*., 1990), the removal of extracellular Ca^{2+} with EGTA induced store depletion in HEK cells (not shown). Therefore, >10 min equilibration was usually required to attain steady-state $[Ca^{2+}]_i$ levels. After prolonged equilibration in a Ca^{2+}-free solution, the $[Ca^{2+}]_i$ levels dropped close to 50 nM. Subsequent equilibration in 500 μM Ca^{2+} restored the basal $[Ca^{2+}]_i$ both in controls and in TRP5-expressing cells to 165.8 ± 42.2 nM (n = 8, Figure 5B). These results suggest that expression of TRP5 does not alter basal $[Ca^{2+}]_i$ levels in HEK cells. A further implication of these results is that the majority of recombinant channels formed by TRP5 are probably not spontaneously active but only become active upon depletion of Ca^{2+} stores, as can be inferred from the experiment shown in Figure 4. Therefore, we analysed $[Ca^{2+}]_i$ levels in cells equilibrated in a nominal Ca^{2+}-free solution after treatment with thapsigargin (Figure 5C). After readdition of 500 μM $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ increased more rapidly and to higher levels in HekCCE2 cells than in controls. When $[Ca^{2+}]_o$ in the solution used in the Ca^{2+} readdition step was varied between 10 μM and 3 mM, the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ relationship in HekCCE2 was much steeper than in control cells (Figure 5D). Additionally, the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ curve of HekCCE2 cells was shifted to concentrations of external Ca^{2+} ~10 times lower when compared with the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ curve of controls. At 3 mM $[Ca^{2+}]_o$, the $[Ca^{2+}]_i$ levels of HekCCE2 cells were 1.7 times higher than in controls. As illustrated in Figure 5E, 3-fold higher $[Ca^{2+}]_i$ levels were also observed after readdition of 3 mM $[Ca^{2+}]_o$ to HekCCE2 cells that were incubated in thapsigargin plus EGTA for 30 min (HekCCE2: 436.1 ± 77.3 nM, n = 8; control cells: 142.8 ± 52.8 nM, n = 12).

Using the $[Ca^{2+}]_i$–$[Ca^{2+}]_o$ curves of Figure 5D, the EC_{50} can be estimated to be ~70 μM $[Ca^{2+}]_o$ and 1 mM $[Ca^{2+}]_o$ for HekCCE2 and control cells, respectively. These EC_{50} values are probably underestimates because no saturation was observed below 3 mM $[Ca^{2+}]_o$, but the EC_{50} of control cells lies within the range reported previously (Takemura and Putney, 1989). The straightforward interpretation of the results of Figure 5D is that expression of TRP5 increased the Ca^{2+} sensitivity of the CCE naturally present in HEK cells in two ways: (i) CCE occurs at lower $[Ca^{2+}]_o$ and (ii) the dependence of CCE on $[Ca^{2+}]_o$ appears to be much steeper than in control cells. Taken together, these results support the findings of the patch–clamp experiments that TRP5 forms capacitative Ca^{2+} channels which are highly Ca^{2+} selective.

Discussion

In this study, we describe the structure of the CCE channel TRP5, the functional properties of the recombinant channel and the expression of its mRNA in excitable cells of the brain. TRP5 shares 68.5% amino acid sequence identity with TRP4 but has significantly lower sequence homology (<48%) to other TRP-related proteins. Figure 6 shows the evolutionary relationship among mammalian TRP homologues. At least three subfamilies can be distinguished, with one subclass comprising TRP5 and TRP4 (Figure 6B). The sequences of the various TRP1 orthologues are the most closely related to TRP5 and TRP4 in terms of their evolutionary distance, whereas TRP3 and TRP6 belong to an independent and less related subclass. TRP2 might be a member of a fourth subclass; however, it has not been functionally expressed so far. This evolutionary classification apparently corresponds to functional features of the channel proteins including their activation mechanisms: TRP1, like TRP5 and TRP4, appears to be activated by Ca^{2+} store depletion, but underlies non-selective cation currents (Zhu *et al*., 1996; Zitt *et al*., 1996), whereas the *trp3* and *trp6* cDNAs encode non-selective cation channels stimulated by G protein-coupled receptors rather than by depletion of intracellular Ca^{2+} stores (Zitt *et al*., 1997; Hurst *et al*., 1998; Zhu *et al*., 1998).

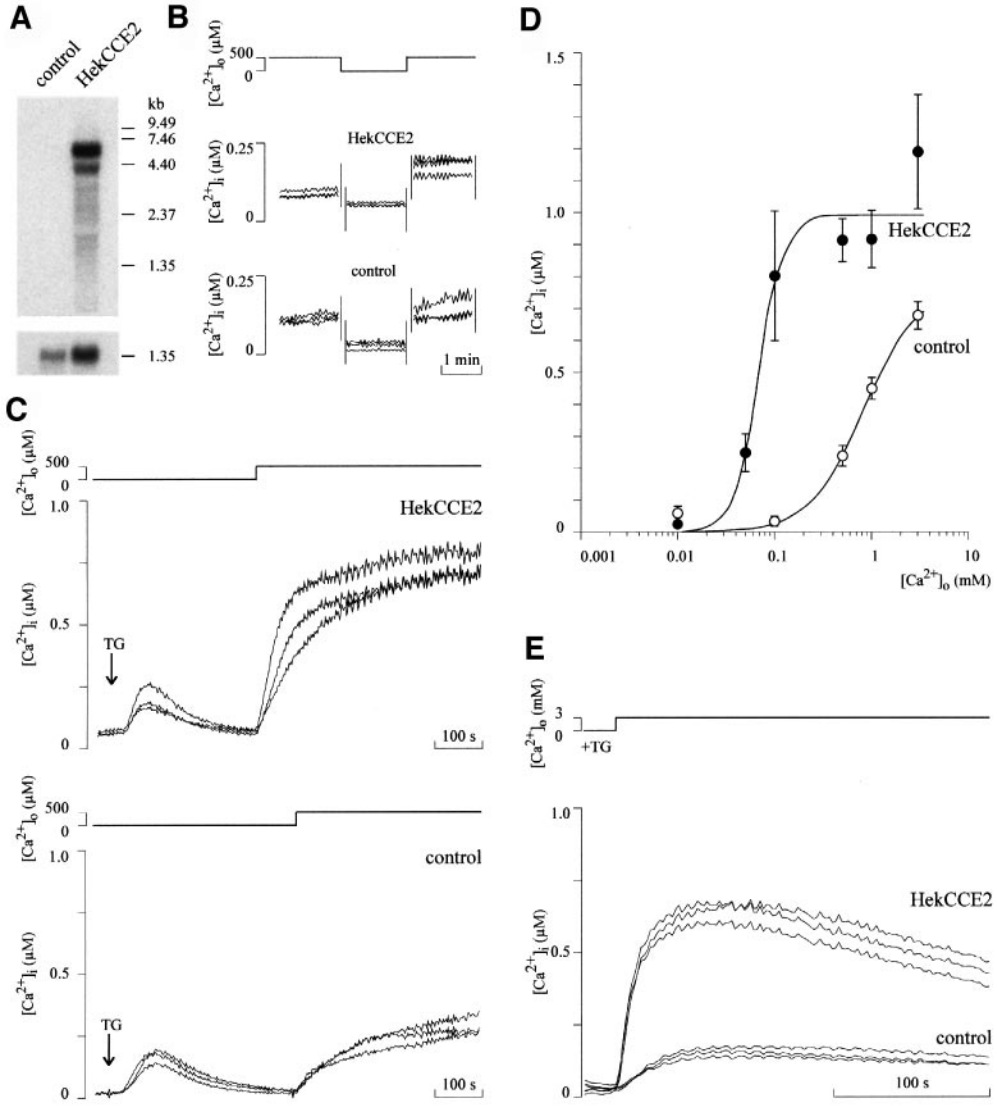

Fig. 5. Store-operated calcium entry in stably TRP5-expressing HEK cells. (**A**) Northern blot analysis of TRP5 transcripts in clone HekCCE2 and non-transfected HEK control cells (upper panel) as well as GAPDH mRNA (lower panel). (**B**) Measurements of steady-state $[Ca^{2+}]_i$ levels in HekCCE2 cells (upper panel) and in control cells (lower panel) equilibrated in solutions containing either 500 μM $[Ca^{2+}]_o$ or no extracellular Ca^{2+} (500 μM EGTA). Broken lines represent 20 min equilibration periods. (**C**) Transient $[Ca^{2+}]_i$ changes after store depletion. Cells were kept in nominal Ca^{2+}-free buffer before 2.5 μM thapsigargin (arrow) was added to deplete intracellular stores. Upon readdition of 500 μM calcium to the bath, the $[Ca^{2+}]_i$ increased in HekCCE2 cells (upper panel) faster and to levels higher than in control cells (lower panel). Representative traces of three independent cells are displayed. (**D**) $[Ca^{2+}]_i$–$[Ca^{2+}]_o$ relationship. $[Ca^{2+}]_i$ was measured 300 s after readmission of various concentrations of external Ca^{2+} in experiments as illustrated in (C) with HekCCE2 cells (●) and control cells (○). Values are given as means ± SEM. (**E**) HekCCE2 and control cells were incubated for 30 min in nominal free extracellular Ca^{2+} in the presence of 5 μM thapsigargin and 2 mM EGTA. At the time indicated, 3 mM Ca^{2+} was introduced into the bath. Representative traces of three independent cells are displayed.

So far, TRP4 and TRP5 are the only members of the family of mammalian TRP homologues possessing the basic functional features of CCE channels, i.e. they appear to be store-operated and they are Ca^{2+} selective. To emphasize these properties, TRP4 and TRP5 might also be called CCE1 (Philipp *et al*., 1996) and CCE2, respectively.

In HEK cells, CCE mediated by TRP5/CCE2 is activated by Ca^{2+} store depletion either by cell dialysis with $InsP_3$ in the presence of 10 mM EGTA or by cell perfusion with thapsigargin. This CCE depends on the $[Ca^{2+}]_o$ and is most obvious at concentrations above 50 μM up to 1 mM. At higher $[Ca^{2+}]_o$, TRP5/CCE2-mediated Ca^{2+} entry might be difficult to distinguish from the CCE endogenous to HEK cells (e.g. at 3 mM $[Ca^{2+}]_o$, Figure 5D). Accordingly, Okada *et al*. (1998) reported that thapsigargin increased $[Ca^{2+}]_i$ in TRP5-transfected HEK293 cells to levels similar to those in control cells at a $[Ca^{2+}]_o$ of 2 mM.

Although TRP5/CCE2 and TRP4/CCE1 are more closely related to each other than to any other of the known mammalian TRP homologues, their C-terminal regions revealed only a few similarities. Nevertheless, these regions account for the overall length of the two proteins, which are significantly longer (974 and 981 amino acid residues) than any of the other mammalian clones reported so far. This may be significant since the C-terminal region seems to confer thapsigargin sensitivity to TRP channels and might, therefore, be involved in linking plasma membrane channels to store depletion (Sinkins *et al*., 1996). Additionally, it has been shown that in *Drosophila* photoreceptor cells, TRP interacts with

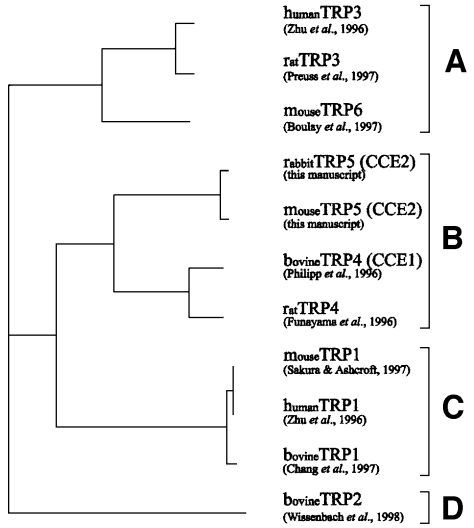

Fig. 6. Phylogenetic tree of mammalian TRP/TRPL homologues. Only complete coding cDNA sequences of mammalian TRP/TRPL homologues have been included. TRPC1 (Wes *et al*., 1995) and TRPC1A (Zitt *et al*., 1996) are variants of hTRP1 apparently encoded by the same gene. The DDBJ/EMBL/GenBank accession No. of bTRP2 is AJ006304.

a PDZ-domain of the INAD protein via its C-terminus (Shieh and Zhu, 1996), integrating the TRP channel into a multi-signalling complex (Huber *et al*., 1996; Chevesich *et al*., 1997; Tsunoda *et al*., 1997). Interestingly, the C-terminal five amino acid residues VTTRL of TRP5 comprise the amino acid motif TXL resembling the known PDZ-domain interaction site S/TXV of other ion channels (Kornau *et al*., 1997). This sequence motif of TRP5 is conserved in the TRP4 protein and may be responsible for binding of TRP4 and TRP5 to mammalian INAD-like proteins, as for example hINADL (Philipp and Flockerzi, 1997).

Recent reports provided evidence for an interaction of TRP and TRPL which might contribute channel subunits to form heteromultimeric channels (Gillo *et al*., 1996; Xu *et al*., 1997). TRP5 may be a subunit of a homo- or a heteromultimeric complex composed of TRP5 and, for example, another mammalian TRP-related protein. At present, the actual subunit arrangement of CCE channels is unknown, and we cannot rule out the possibility that endogenous proteins may also contribute to the new currents detected in TRP5-transfected cells. As shown in Figure 3, formation of heteromultimeric TRP5–TRP4 channels may occur in CA1 pyramidal neurons of the hippocampus. However, the specific expression of both genes in different cells and tissues implies that TRP5 and TRP4 function as store-operated Ca^{2+} channels independently of each other.

TRP5 transcripts were also detected in the olfactory bulb and the lateral cerebellar nuclei. So far, CCE in neurons has been implicated to be involved in the migration of growth cones (Gomez *et al*., 1995) but, in contrast to its well-established functional roles in non-excitable cells, little is known about its contribution to neuronal Ca^{2+} homeostasis. In pyramidal neurons, refilling of ryanodine-sensitive stores requires a transmembrane Ca^{2+} influx pathway that is active at the resting membrane potential (Garaschuk *et al*., 1997). To account for this Ca^{2+} influx, the existence of a Ca^{2+} entry pathway in the plasma membrane has been hypothesized (Garaschuk *et al*., 1997) which is activated by depletion of ryanodine-sensitive Ca^{2+} stores. This mechanism would be analogous to the CCE, activated by agonist-induced depletion of $InsP_3$-sensitive Ca^{2+} stores.

In conclusion, we have shown that TRP5 is activated by Ca^{2+} store depletion and, therefore, is responsible for CCE. The recombinant TRP5 currents display inward going rectification and are reversed at positive potentials, indicating that TRP5 channels are calcium selective. Accordingly, expression of TRP5 increase the Ca^{2+} sensitivity of CCE in the host cells. The cloning of TRP5 and the characterization of its function and expression pattern in the brain will facilitate efforts to identify the role of CCE in excitable cells.

Materials and methods

Cloning of TRP5 from rabbit brain (rTRP5) and mouse brain (mTRP5)

Using degenerate oligonucleotide primers corresponding to conserved amino acid regions in TRP and TRPL (W^{644}GLLMFG and E^{682}WKFART, numbering according to TRPL), the 133 bp cDNA fragment C (nucleotides 1799–1931, Figure 1A and B) was amplified from rabbit brain poly(A)$^+$ RNA by RT–PCR. This fragment showed significant amino acid sequence homology to TRP and TRPL and was used as a probe to screen an oligo(dT)-primed cDNA library from rabbit brain. Three independent clones were obtained, including N45 (nucleotides 1584–3773, Figure 1A). The 5′ nucleotide sequence was identified by screening a specifically primed rabbit brain cDNA library using a synthetic primer complementary to nucleotides 1886–1867 for reverse transcription and a cDNA fragment covering nucleotides 1584–1718 as probe. Eight independent clones were analysed, including clone M14 (Figure 1A). All cDNA clones obtained revealed sequences identical within the protein coding region. All cDNAs were sequenced on both strands using a laser fluorescence DNA sequencer. To obtain the cDNA sequence of mTRP5, two degenerate oligonucleotides corresponding to amino acids M^1AQLYYKK (5′-ATG GCN CAR YTN TAY TAY AAR AA-3′) and M^{960}HKWGDG (5′-CCR TCN CCC CAY TTR TGC AT-3′) of rTRP5, respectively, were used to amplify a 2897 bp cDNA fragment which was subcloned. Four independent cDNA clones were sequenced on both strands. The nucleotide sequences of rTRP5 and mTRP5 cDNA have been deposited in DDBJ/EMBL/GenBank under the accession Nos AJ006203 and AJ006204, respectively.

Northern blot analysis

Northern blot analysis was performed as described (Philipp *et al.*,1996). The probe was the –1 to 2571 nucleotide fragment spanning most of the protein coding region of rTRP5 (Figure 1A) labelled by random priming with [α-^{32}P]dCTP. For analysis of rabbit tissues,10 μg of poly(A)$^+$ RNA were applied. A human brain multiple tissue Northern blot (Clontech) was hybridized under the same conditions but at 37°C. As a control for the integrity and amount of the transferred RNA, all filters were stripped and rehybridized with a 239 bp cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Filters were exposed to X-ray films for 9 days (TRP5 probe), 3 days (GAPDH probe, rabbit tissues) or 2 h (GAPDH probe, human tissues).

In situ hybridization

Sections (8 μm thick) from freshly frozen adult mouse brains were mounted on silane-coated glass slides and immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; Sambrook *et al*., 1989). After two washing steps in PBS for 5 min, the sections were dehydrated through a series of 2 min incubations in 30, 60, 80, 95 and 100% ethanol, air dried and stored in desiccant at –80°C. For hybridization, slides were rehydrated and treated with 1 μg/ml proteinase K in 100 mM Tris–HCl, 50 mM EDTA (pH 7.5) at 37°C for 30 min. After refixation in 4% PFA/PBS, the specimens were immersed in 100 mM triethanolamine-HCl (pH 8.0) and twice in 0.25% acetic anhydride/0.1 M triethanolamine-HCl (pH 8.0), dehydrated and air dried. As a probe, we subcloned a 1497 bp cDNA fragment corresponding to amino

acids 467–965 of mTRP5 (Figure 1B) in pBluescript. As a probe for detection of mouse TRP4, we used a 528 bp cDNA fragment amplified with primers 5′-ACA GTG ATC TGA ACC CAC GG-3′ (Freichel *et al*., 1998) and 5′-ATT CTA TCT GCA TGG TCG GC-3′ (Petersen *et al*., 1995) covering part of the hydrophobic core region of mTRP4. Sense and antisense RNA probes were synthesized with SP6 and T7 polymerase, respectively, in the presence of [α-^{33}P]UTP using the linearized recombinant plasmid. The cRNA probes were purified by gel chromatography, and the probe length was adjusted to 200 bases by alkaline treatment. The probes were precipitated and dissolved at a concentration of 60 ng/ml of hybridization solution containing 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.5 mg/ml yeast tRNA, 10% dextran sulfate and buffer A [10 mM dithiothreitol (DTT), 50% formamide, 30 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, 5 mM EDTA, 20 mM Tris–HCl, pH 6.8]. Hybridization was carried out with 8 μl of solution per section. Sections were covered with parafilm, incubated in a moist chamber at 55°C overnight followed by sequentially rinsing the slides twice in buffer A at 55°C for 60 min, twice in 0.5 M NaCl, 10 mM Tris–HCl, 5 mM EDTA pH 7.5 (NTE) at 37°C for 15 min and once in 20 μg/ml of RNase A in NTE at 37°C for 30 min. After a further 15 min incubation in NTE at 37°C and 60 min in buffer A at 55°C, the slides were washed twice at room temperature in 2× SSC, and once at 65°C and once at room temperature in 0.1× SSC for 15 min each. Finally, the sections were dehydrated through a graded series of ethanol containing 300 mM ammonium acetate followed by two washes in 100% ethanol and air drying. After exposure to an X-ray film for 5 days, the slides were dipped in Kodak NTB-2 emulsion and exposed for 3 weeks. Sections were stained with haematoxylin and eosin and photographed using a Contax 167 MT camera adapted to a Zeiss Axioskope microscope.

Construction of the dicistronic TRP5/GFP expression plasmid, transient transfection and selection of stable TRP5-expressing HEK cells

To obtain the recombinant dicistronic expression plasmid pdiCCE2 carrying the entire protein coding regions of rTRP5 and the GFP (Prasher *et al*., 1992), the 5′- and 3′-untranslated sequences of the rTRP5 cDNA were removed, the consensus sequence for initiation of translation in vertebrates (Kozak, 1987) was introduced immediately 5′ of the translation initiation codon and the resulting cDNA was subcloned in pcDNA3 downstream of the cytomegalovirus promoter. The IRES derived from encephalomyocarditis virus (Mountford *et al*., 1994) followed by the GFP cDNA containing a Ser65Thr mutation (Heim *et al*., 1995) was then cloned 3′ to the rTRP5 cDNA. When indicated, the same plasmid containing the lacZ cDNA instead of the TRP5 cDNA was used as control (pdiLacZ).

For transient expression of TRP5, HEK cells (ATCC CRL 1573) were transfected with pdiCCE2 using lipofectamine (Lifetechnologies) as described previously (Philipp *et al*., 1996). Electrophysiological recordings of cells showing green fluorescence were performed 48–72 h post-transfection. Stable cell lines including HekCCE2 were selected by addition of 400 μg/ml geneticin and by their green fluorescence.

Electrophysiological recordings

HEK cells transfected with pdiCCE2 were detected by development of green fluorescence. As controls, either non-transfected HEK cells or pdiLacZ mock-transfected cells were used. Single cells were voltage-clamped in the whole-cell mode (Hamill *et al*., 1981) using an EPC-9 (HEKA) patch–clamp amplifier as described (Philipp *et al*., 1996). The pipette solution contained 115 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH 7.4 adjusted with CsOH). The bath solution contained 115 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM HEPES (pH 7.4 adjusted with NaOH). Whole-cell currents were elicited by 250 ms voltage-clamp ramps from –100 mV to + 60 mV every 5 s (0.64V/s). For calculation of current densities, the membrane capacitance (C_m) was read from the settings provided by the amplifier after automatic cancellation of transients occurring in the whole-cell mode. Inward and outward currents were evaluated as the mean current amplitude measured within a 10 mV window placed at –80 mV and +40 mV, respectively. Data are given as means ± SD.

Measurements of $[Ca^{2+}]_i$ in stable transformed HEK cells

Measurements of $[Ca^{2+}]_i$ in single HEK cells were performed with a digital imaging system (T.I.L.L. Photonics). Cells grown on coverslips for 3 days were loaded with 1 μM fura-2/AM (Molecular Probes) for 30 min at 37°C in minimal essential medium containing 10% fetal calf serum. Cells were washed three times with 300 μl of a buffer containing 115 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES (pH 7.4). The extracellular Ca^{2+} concentration was varied between 10 μM and 3 mM. Nominal Ca^{2+}-free solutions contained ~2 μM $[Ca^{2+}]_o$. In some experiments, EGTA was added to the external solution at the concentrations given in the text. $[Ca^{2+}]_i$ was calculated from fluorescence ratios obtained at 340 and 380 nm excitation wavelengths essentially as described (Garcia *et al*., 1994). Experiments were repeated three times, with measurements of three cells in each experiment. A modified protocol was applied (Zitt *et al*., 1996) in some experiments (Figure 5E) in which eight cells were measured in three independent assays. Unless otherwise stated, values are given as means ± SD.

Miscellaneous methods

Sequences were analysed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) of the biocomputing unit at the German Cancer Research Centre Heidelberg. The phylogenetic distances of proteins were calculated with the clustal/clustree program (Saitou and Nei, 1987; Thompson *et al*., 1994), and the similarity of protein sequences in pairs with the bestfit program (Needleman and Wunsch, 1970). Photographs were scanned and processed using Corel Photo-Paint/Corel Draw and Adobe PhotoShop.

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