Degeneration of an Intracellular Ion Channel in the Primate Lineage by Relaxation of Selective Constraints

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Abstract

Ion channel genes are highly conserved and are rarely degenerated in the primate lineage leading to humans. So far, the only well-characterized ion channel known to be degenerated in primates is the plasma membrane transient receptor potential channel TRPC2, possibly due to changes in the pheromone signaling. Here, by analyzing the sequence data from ten primate species, we have determined the degeneration process of the *TPC3* gene that encodes a member of the two-pore channel (TPC) family recently implicated in Ca^{2+} release by nicotinic acid adenine dinucleotide phosphate from intracellular acidic stores in animals. We show that degeneration of *TPC3* likely began in the common ancestors of Apes and Old World monkeys through a conserved inactivating mutation, followed by additional deleterious mutations resulting in the generation of a *TPC3* pseudogene in the descendant catarrhine lineage. Located at a chromosome recombination hot spot, catarrhine *TPC3* pseudogenes underwent a series of lineage-specific rearrangements, including exon deletion and duplication. In contrast, we identify near full-length *TPC3* sequences in New World monkeys and Prosimians and show that the gene is subjected to strong purifying selection and therefore likely functional. Our data provide the first evidence for relaxed functional constraints for an intracellular ion channel in primates and shed novel insights into the evolution and regulation of Ca^{2+} signaling in the primate lineage.

Key words: Ca^{2+} signaling, degeneration, NAADP, primates, pseudogene, two-pore channel.

Introduction

In the primate lineage leading to humans, ion channel genes are normally preserved, and deleterious mutations underlie many severe genetic diseases, such as fatal cardiac arrhythmias, cystic fibrosis, and ataxia (Hubner and Jentsch 2002). Among the many different ions regulated by ion channels, Ca^{2+} ions play a particularly important signaling role in modulating numerous physiological processes in almost all types of cells (Berridge et al. 2003; Clapham 2007). Cytosolic Ca^{2+} concentration is tightly regulated by various Ca^{2+} channels, exchangers, and pumps, most of which are highly conserved during metazoan evolution, from the unicellular ancestor of metazoans, *Monosiga brevicollis*, to mammals (Cai 2008). Cytosolic Ca^{2+} levels can be raised by Ca^{2+} influx through a variety of Ca^{2+} permeable channels in the plasma membrane and Ca^{2+} release through intracellular channels in the membranes of Ca^{2+} stores (fig. 1).

Primate lineage loss of ion channel genes is a very rare phenomenon likely associated with weakening or elimination of related selective constraints (Liman 2006). So far, the only well-characterized ion channel pseudogene in the primate lineage is the *TRPC2* gene belonging to the transient receptor potential (TRP) channel gene family (Ramsey et al. 2006). Rodent TRPC2 channels are fully functional at the plasma membrane, inducing sustained Ca^{2+} influx during the sperm acrosomal reaction (Vannier et al. 1999; Jungnickel et al. 2001). Rodent TRPC2 channels are also critical for the pheromone signaling in the vomeronasal organ (Liman et al. 1999). Eight years after the original identification of the human *TRPC2* pseudogene (Wes et al. 1995), evolutionary studies in primates revealed that *TRPC2* is preserved in New World monkeys but had degenerated in the ancestor of Old World monkeys and Apes (catarrhines) (Liman and Innan 2003; Zhang and Webb 2003). It is speculated that in catarrhine primates, visual and auditory signals might have largely replaced pheromone signals for communicating social and reproductive status. Relaxation of selective pressure in catarrhine primates likely led to the degeneration of *TRPC2* with inactivating mutations (Liman and Innan 2003; Zhang and Webb 2003).

Activation of intracellular Ca^{2+} channels is gated by second messengers, such as inositol 1,4,5-trisphosphate, cyclic adenosine diphosphate ribose, and nicotinic acid adenine dinucleotide phosphate (NAADP) (Berridge et al. 2003; Clapham 2007). First discovered in sea urchin egg homogenates two decades ago (Clapper et al. 1987), NAADP is believed to be the most potent endogenous Ca^{2+}-mobilizing agent identified so far, mediating diverse cellular functions, such as fertilization, insulin secretion, and T lymphocyte activation (Guse and Lee 2008). In many cell types, NAADP is highly unusual in that it appears to release calcium from acidic organelles (Patel and Docampo 2010). However, the molecular identity of the channel targeted by NAADP has remained obscure (Guse 2009).

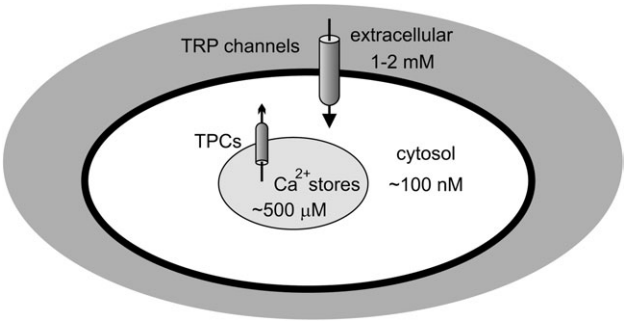

Fig. 1. Both Ca^{2+} influx and Ca^{2+} release contribute to Ca^{2+} signaling. Ca^{2+} mainly derives from two sources—Ca^{2+} influx from extracellular spaces, mediated by the plasma membrane Ca^{2+} permeable channels, such as TRP channels, and Ca^{2+} release from Ca^{2+} stores through intracellular channels, such as TPCs. Ca^{2+} gradients across these membranes, shown by the direction of arrows, are very steep, as reflected in the differences in relative Ca^{2+} concentrations.

Recently, we (Brailoiu et al. 2009, 2010) and others (Calcraft et al. 2009; Zong et al. 2009) have identified the two-pore channels (TPCs) as a novel family of ion channels underlying NAADP-sensitive Ca^{2+} release in animals. All three members of the animal TPC family localize to acidic organelles, such as endosomes and lysosomes, and induce Ca^{2+} release from these acidic Ca^{2+} stores in response to NAADP (Brailoiu et al. 2009, 2010; Calcraft et al. 2009; Zong et al. 2009). TPCs comprise two repeats of six transmembrane regions and might reflect the evolutionary link between one repeat voltage–gated K^+ channels and four repeat voltage–gated Na^+ and Ca^{2+} channels (Ishibashi et al. 2000). The identification of animal TPCs as a novel class of NAADP-sensitive endolysosomal ion channels adds a new dimension into our current understanding of Ca^{2+} signaling in human physiology (Galione et al. 2009; Patel et al. 2010).

Evolutionary genomics has become a powerful approach to decipher the Ca^{2+} signaling pathway in a lineage- and species-specific manner at the genomic scale (Cai 2007a, 2007b; Case et al. 2007; Cai 2008; Cai and Clapham 2008). Our recent genomic analysis of TPCs provides direct evidence that *TPC3* is a pseudogene in human, chimpanzee, and monkey genomes but not in dog, cattle, and horse genomes (Brailoiu et al. 2009, 2010). These findings prompted us to further characterize the evolutionary patterns of *TPC3* in primates in order to advance our understanding of the regulation of Ca^{2+} signaling during primate evolution, which is at present poorly understood. Here, we demonstrate that TPC3-mediated NAADP signaling pathway is likely functional in New World monkeys and Prosimians but has been impaired later in the common ancestor of Old World monkeys and apes, resulting from relaxed selective constraints and a conserved inactivating mutation.

Materials and Methods

Database Searches, Multiple Sequence Alignments, and Phylogenetic Analysis

DNA sequences of chicken, dog, and bovine TPC3 were used to perform Blast (Altschul et al. 1997) and BLAT (Kent 2002) searches on the genomic and trace archive databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) and the Ensembl genome Web site (http://www.ensembl.org/). Trace chromatogram data were manually examined for matching sequences. The collected data sets were then subjected to sequence alignments, manual editing, exon structure prediction and comparison, and phylogenetic analysis, essentially as previously described (Cai and Lytton 2004; Cai and Zhang 2006).

To preserve the reading frame and allow for frameshift errors, the Wise2 form (http://www.ebi.ac.uk/Tools/Wise2/index.html) was employed for the translation of TPC3 pseudogenes by comparison with dog and bovine TPC3 protein sequences, followed by manual editing.

Estimation of Nonsynonymous (d_N) to Synonymous (d_S) Nucleotide Substitution Ratio

Codon alignments of TPC3 sequences after removing frameshifts, in-frame stop codons, and gaps were extracted from the PAL2NAL server (Suyama et al. 2006) by using corresponding protein sequence alignments. Estimates of d_N and d_S values were then calculated with the codeml program implemented in the PAML package (Yang 2007).

Results and Discussion

Lineage-Specific Evolution of TPCs in Placental Mammals

The diverse physiological function of NAADP-mediated Ca^{2+} signaling (Guse and Lee 2008) implicates the importance of TPCs in animal physiology. The development of all three TPC members occurred early in metazoan evolution, as shown in the primitive Eumetazoan *Nematostella vectensis* (Brailoiu et al. 2009). Within the mammalian lineage, the number of genes encoding functional ion channels and transporters in each genome appear to be quite stable (Anderson and Greenberg 2001; Cai 2007b; On et al. 2008; Rosati and McKinnon 2009). As one of the few exceptions, the loss or degeneration of *TPC3* has been observed in one group of the placental mammals, Euarchontoglires, but not in its sister group Laurasiatheria (Brailoiu et al. 2010). *TPC3* was completely deleted in mouse and rat genomes, whereas in the three primate genomes, partial *TPC3* pseudogene sequences with exon structure similar to dog and bovine *TPC3* were identified.

To determine whether the changes in *TPC3* occurred in the common ancestors of Euarchontoglires after diverging from Laurasiatheria ~85 Ma (Murphy et al. 2001), we searched for *TPC3* sequences in additional Euarchontoglires genomes. As shown in figure 2A, the *TPC3* gene had degenerated in the two subgroups of Euarchontoglires—Glires and Euarchonta. However, no inactivating mutation of *TPC3* was found in rabbit and guinea pig (Glires) and tree shrew (Euarchonta). Therefore, the degeneration of *TPC3* in rodents and higher primates might be derived from different trajectory of physiological evolution and regulation of the endolysosomal Ca^{2+} signaling pathway. Similarly, in

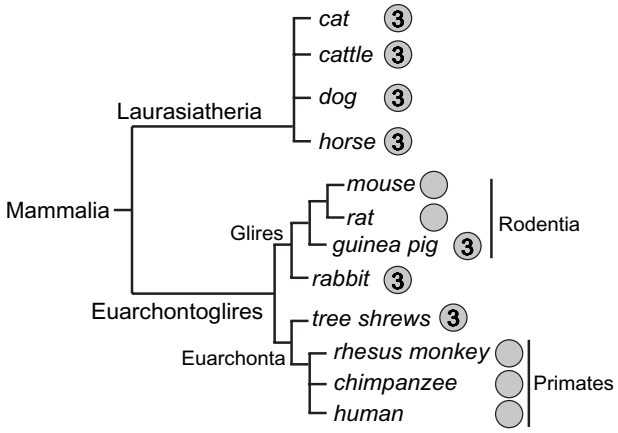

FIG. 2. *TPC3* degeneration occurred independently in the two subgroups of Euarchontoglires—Glires and Euarchonta. Phylogeny of the placental mammals is derived from the Tree of Life project (http://tolweb.org). *TPC3* is depicted as a numbered gray circle. Nonnumbered gray squares indicate the absence or degeneration of *TPC3* in the corresponding genomes.

addition to its degeneration in catarrhine primates (Liman and Innan 2003; Zhang and Webb 2003), *TRPC2* has also degenerated in select mammals, possibly arising from diverse and different ecological correlates (Young et al. 2010).

Molecular Basis for *TPC3* Degeneration in Catarrhines

Primates such as the present day catarrhines (Apes and Old World monkeys), New World monkeys, and Prosimians are thought to have diverged from a common ancestor ~63 Ma (fig. 3*A*) (Goodman et al. 1998). To gain further insight into the *TPC3* gene in this lineage, we identified TPC3 coding sequences from ten available primate genomes and trace archives representing all four infraorders—Lemuriformes, Lorisiformes, Tarsiiformes, and Simiiformes (fig. 3*A*).

The full-length sea urchin (Brailoiu et al. 2010) and chicken (Calcraft et al. 2009) as well as horse, bovine, and dog TPC3 genes contain 19 protein-coding exons. In contrast, the genomes of humans (fig. 3*B* and *C*) and three other Apes (*Pan troglodytes*, *Gorilla gorilla*, and *Pongo pygmaeus*) contain only the protein-coding exons 1–8. *TPC3* sequences from the Old World monkeys *Macaca mulatta* (fig. 3*B* and *C*) and *Papio hamadryas* appear to be more conserved with 16 remaining protein-coding exons (exons 4–19).

All identified sequences possess multiple frameshift or nonsense mutations (fig. 4*A* and supplementary fig. S1, Supplementary Material online). Within the conserved exons (exons 4–8) of *TPC3* between Apes and Old World monkeys, two premature stop codons and one 1-bp deletion were found in Apes and two 1-bp deletions in Old World monkeys. Among these mutations, a single 1-bp deletion (fig. 4*A* and supplementary fig. S1, Supplementary Material online) was shared by all catarrhines, suggesting that it might be one of the earliest inactivating mutations occurring in the common ancestors of Apes and Old World monkeys. This deletion results in a frameshift in the beginning of the putative transmembrane segment (TMS) 5, which would render the TPC3 channel inactive. Indeed, mutation of a single amino acid Leu273 (also conserved in TPC3) in the putative pore region located between TMS5 and TMS6 of human TPC1 completely abolishes NAADP-induced Ca^{2+} signals (Brailoiu et al. 2009) (fig. 4*B*). Other mutations in exons 4–8 were likely accumulated at different time points in the descendant lineages.

In marked contrast to the primate *TRPC2* pseudogenes, which have preserved the exon structure of their functional counterparts (Liman and Innan 2003; Zhang and Webb 2003), *TPC3* pseudogenes in primates show patterns of dynamic local recombination, including exon deletion and duplication (fig. 3*C*). In *Homo sapiens*, three duplicated fragments (corresponding to exons 1–8) were identified, one of which was in the opposite orientation (fig. 3*C*). In *M. mulatta TPC3*, we found flanking fragments corresponding to exons 5–8, 4–18, and 17–19, with exons 5–8 in the opposite orientation. Interestingly, human *TPC3* is located at chromosomal position 2q13, one of the recombination hot spots associated with genome instability and genetic disease (Bailey et al. 2002; Rudd et al. 2009). In addition, 2q13 is also the fusion site for the formation of chromosome 2 by head-to-head combination of two acrocentric chromosomes in the human lineage of evolution (Bailey et al. 2002). Given the conserved nature of the inactivating mutations in *TPC3*, these local recombination events likely occurred more recently.

TPC3 Is Intact in Prosimians and New World Monkeys

To ascertain at what stage TPC3 degeneration began in primate evolution, we examined the genomes of New World monkeys and Prosimians. We were able to identify 19 protein-coding exons for *TPC3* from the New World monkey (*Callithrix jacchus*) (fig. 3*C*). We also identified 15 protein-coding exons from the tarsiers, *Tarsius syrichta*, and 19 and 17 protein-coding exons for two nontarsier Prosimians, *Otolemur garnettii* and *Microcebus murinus*, respectively (supplementary figs. S1 and S2, Supplementary Material online). Intriguingly, as shown in supplementary figure S1 (Supplementary Material online) and figure 4, no deleterious mutations were found in the TPC3 sequences from New World monkeys and Prosimians. These data suggest that TPC3 gene is likely functional in these primate lineages. Thus, we provide the first evidence that the degeneration of *TPC3* occurred in the common ancestors of Apes and Old World monkeys ~25–40 Ma (Goodman et al. 1998).

Relaxed Selective Pressure on *TPC3* during the Primate Evolution

Negative (or purifying) selective pressure on functional genes prevents the spread of deleterious mutations. In some cases, due to changes in environmental signals, deleterious mutations may not necessarily affect the fitness of the organism and may be accumulated (Nei 2005). For instance, the degeneration of *TRPC2* in catarrhines might have resulted from the replacement of pheromone

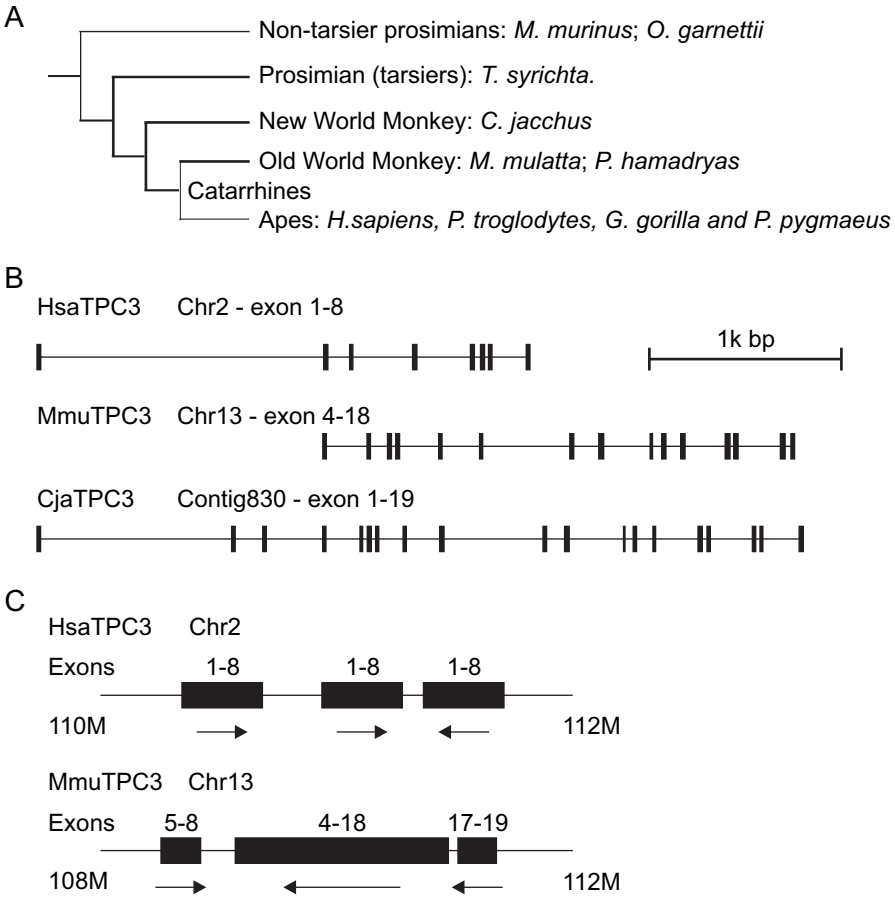

Fig. 3. Structure of the *TPC3* gene in representative primates. (*A*) Phylogenetic tree of the ten primate species described in this study is based on the Tree of Life project, which is also confirmed by the maximum likelihood analysis (Felsenstein 1996) using TPC3 sequences. (*B*) Exon structure of TPC3 from *Homo sapiens* (*Hsa*), *Macaca mulatta* (*Mmu*), and *Callithrix jacchus* (*Cja*). Their genomic localizations and exon numbers are also indicated. (*C*) *TPC3* pseudogenes display lineage-specific rearrangements. Shown are the pseudogene fragments with corresponding exon numbers. The direction of arrows indicates the orientation of the exons.

signaling by color vision (Liman and Innan 2003; Zhang and Webb 2003).

Comparison of the number of nonsynonymous substitutions per nonsynonymous site (d_N) and synonymous substitutions per synonymous site (d_S) of protein-coding genes is often used for detecting the presence or absence of selective pressure (Nei 2005). If a functional gene is subjected to purifying selection pressure, deleterious nonsynonymous substitutions are likely prevented from fixation but synonymous substitutions may be tolerated, leading to a d_N/d_S ratio <1. In the absence of selective pressure, a pseudogene is evolving neutrally with approximately equal nonsynonymous and synonymous substitutions, resulting in a d_N/d_S ratio ≈ 1.

To test whether changes in selective pressure concurred with the appearance of *TPC3* pseudogenes in primates, we estimated the maximum likelihood ratio of d_N and d_S of primate *TPC3* sequences. We first generated a codon alignment by using TPC3 sequences from all ten primates. The resulting alignment contains 573 bp after removing frameshifts, in-frame stop codons, and gaps (supplementary fig. S1, Supplementary Material online). Consistent with the presence of functional TPC3 channels in their genomes, branches leading to Prosimians and New World monkeys show d_N/d_S ratios significantly lower than 1 ($P < 0.0001$), suggesting strong purifying selection pressure (fig. 5*A*). Purifying selection is also evident for full-length mammalian TPC3 channels in Glires (rabbit vs. guinea pig, d_N/d_S ratio 0.1574, $P < 0.0001$) and Laurasiatheria (bovine vs. dog, d_N/d_S ratio 0.2050, $P < 0.0001$). In contrast, all branches leading to and within the catarrhines exhibit relaxed selective constraints of TPC3 with a mean d_N/d_S ratio 0.9962 ($P > 0.05$) (fig. 5*A*).

The above analysis was based on a common but relatively short codon alignment restricted to regions encoding the partial N-terminal half of TPC3 (supplementary fig. S1, Supplementary Material online). This may limit the power to accurately assess the evolution of complete TPC3. For instance, although the branch connecting New World monkey (*C. jacchus*) with its nearest ancestor displays strong selection (mean d_N/d_S ratio 0.0633, $P < 0.0001$), the d_N/d_S ratio for *C. jacchus* is relatively high (0.5358, $P = 0.0855$). Conversely, the branch connecting Old World monkeys and the common ancestor of catarrhines displays neutral evolution (mean d_N/d_S ratio 0.9962, $P > 0.05$), the d_N/d_S ratio for *M. mulatta* is relatively low (0.2302,

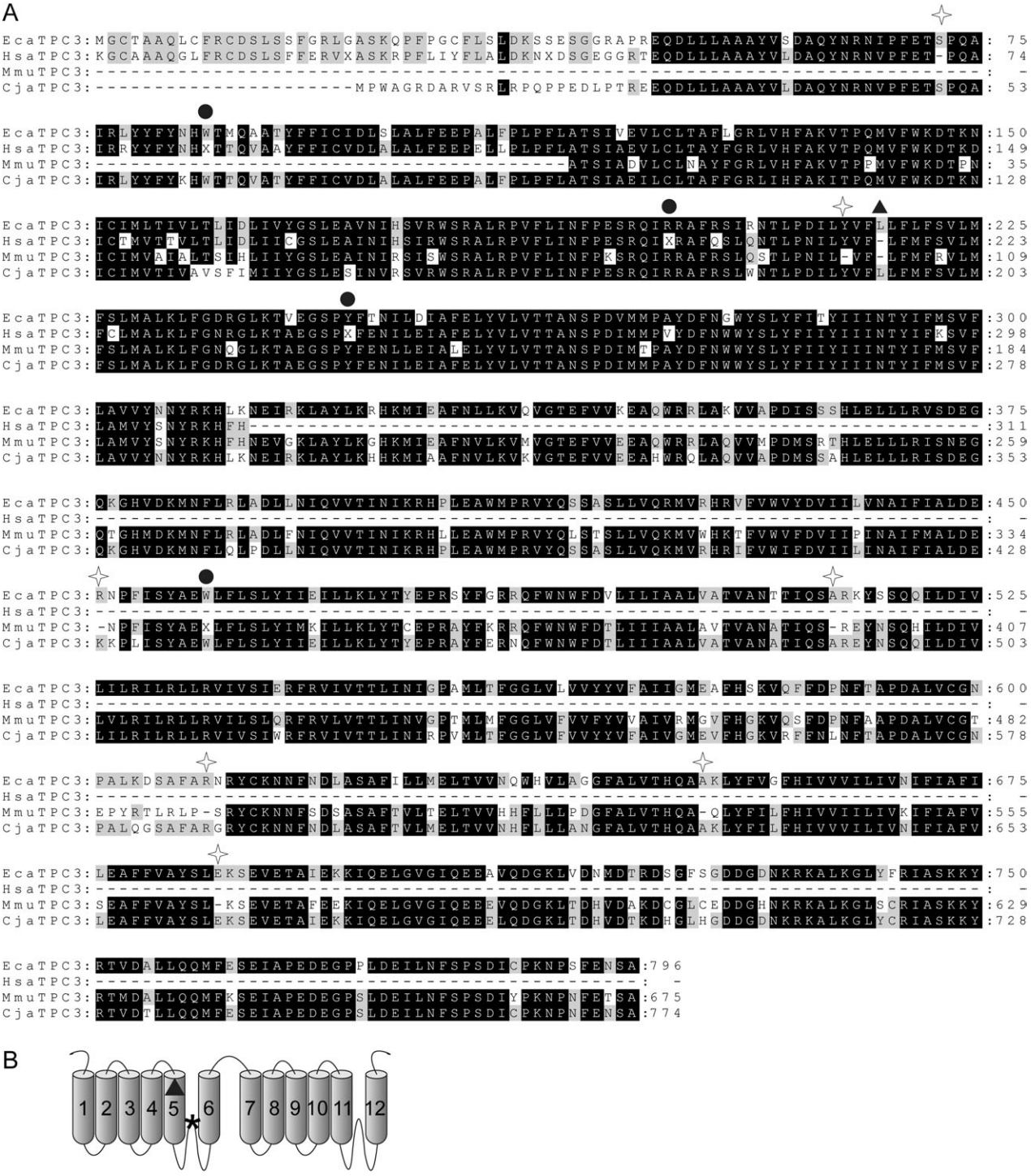

Fig. 4. TPC3 sequence alignment of select primate species reveals deleterious mutations in Apes and Old World monkeys. (*A*) The deduced TPC3 amino acid sequences of Apes (*H. Sapiens*, *Hsa*), Old World monkeys (*Macaca mulatta*, *Mmu*), and New World monkeys (*Callithrix jacchus*, *Cja*) were aligned with the horse TPC3 sequence (*Equus caballus*, Eca). Nucleotide deletions or insertions are marked with stars, and in-frame stop codons are shown with filled circles above the aligned sequences. The deletion mutation conserved between Apes and Old World monkeys are indicated by a filled triangle. Supplementary figure S1 (Supplementary Material online) contains the complete alignments for all primate TPC3 sequences. (*B*) A topological model of TPC3 with 12 putative transmembrane regions, showing the location of a conserved deletion mutation ("filled triangle") in catarrhine TPC3s and a Leu residue ("asterisk") essential for TPC activity (conserved as Leu273 in HsaTPC1 [Brailoiu et al. 2009]).

$P = 0.1478$). We therefore estimated the d_N/d_S ratio of the six primate *TPC3* sequences for which more complete coverage was available (fig. 5*B*). The resulting codon alignment contains 1,284 bp extending throughout the coding sequence (supplementary fig. S1, Supplementary Material online). Consistently, only TPC3 sequences in Old World monkeys show relaxation of functional constraint (mean d_N/d_S ratio 1.2231, $P > 0.05$), whereas other primate

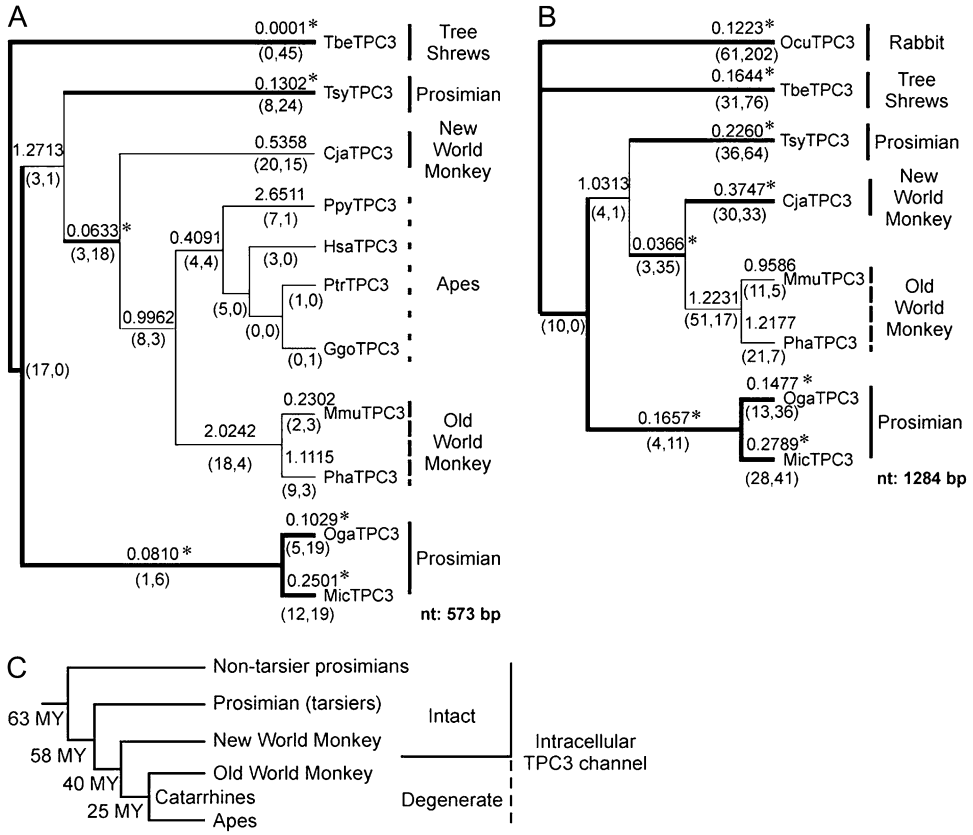

Fig. 5. Maximum likelihood estimates of d_N/d_S ratios indicate the loss of selective pressure in the primate lineage. (*A*) Estimates of d_N/d_S ratios using ten primate TPC3 sequences. (*B*) Estimates of d_N/d_S ratios based on six non-Apes primate TPC3 sequences. The length of codon alignment is shown at the bottom. The tree branch lengths of the primate phylogeny are not proportional in scales. d_N/d_S ratios are shown above the branch. The numbers of nonsynonymous and synonymous substitutions are shown consecutively in the brackets below the branch—d_N/d_S ratios are omitted if one of the numbers is "0." Asterisks and thick branches indicate d_N/d_S ratios that are not compatible with neutral evolution ($P < 0.001$). The intermittent lines reflect the degree of exon deletions in the TPC3 sequences. (*C*) The degeneration of the intracellular TPC3 channel occurred in the common ancestors of catarrhines. The divergence times are based on estimates in Goodman et al. (1998). Abbreviations used for primates: *Cja*, *Callithrix jacchus*; *Gga*, *Gorilla gorilla*; *Hsa*, *Homo sapiens*; *Mic*, *Microcebus murinus*; *Mmu*, *Macaca mulatta*; *Oga*, *Otolemur garnettii*; *Pha*, *Papio hamadryas*; *Ppy*, *Pongo pygmaeus*; *Ptr*, *Pan troglodytes*; *Tsy*, *Tarsius syrichta*. Abbreviations for nonprimate mammals: *Ocu*, *Oryctolagus cuniculus*; *Tbe*, *Tupaia belangeri*.

TPC3 sequences are under purifying selection. Taken together, analysis of d_N/d_S ratio provides independent support for the hypothesis that TPC3 channels in Prosimians and New World monkeys are likely functional and that the degeneration of *TPC3* occurred in the common ancestors of Apes and Old World monkeys (fig. 5C).

TPC3 Degeneration and Evolution of Ca^{2+} Signaling in Catarrhines

As studies on the role of TPC channels in NAADP-evoked Ca^{2+} signaling were reported only very recently (Brailoiu et al. 2009, 2010; Calcraft et al. 2009; Zong et al. 2009), little is known about the contribution of individual TPC channel, especially TPC3, at the physiological level. One possibility is that TPC1 and/or TPC2 had replaced TPC3 in the endolysosomal Ca^{2+} signaling pathway, and thus, TPC3 was degenerated. In that case, one would expect to see accelerated evolution of catarrhine TPC1 and/or TPC2 (manifest as an increase in d_N/d_S ratios) in order for them to adapt to newly acquired functions. However, TPC1 and TPC2 in humans are under stringent purifying selection relative to cows (which possess all three TPC genes) with d_N/d_S ratio 0.0708 for human TPC1 versus bovine TPC1 ($P < 0.0001$) and 0.1493 for human TPC2 versus bovine TPC2 ($P < 0.0001$). Therefore, it is unlikely that TPC1 and/or TPC2 expanded their function to replace TPC3 in the recent primate lineages.

Alternatively, the degeneration of catarrhine *TPC3* might be caused by changes of environmental signals involved in TPC3-specific NAADP-induced endolysosomal Ca^{2+} signaling. Sensory signaling in primates such as pheromone, vision, smell, and taste is constantly adapting to new environmental changes and physiological requirements. In addition to TRPC2, pheromone receptors and odorant receptors also appear to be subjected to relaxed selective force in humans (Liman 2006). Interestingly, the partial human *TPC3* pseudogene sequence is detected in an uncharacterized, widely expressed, LIN-11, Isl1 and MEC-3-like domain expressed sequence tag cluster (UniGene—Hs.535619), with the most abundant expression in the

reproductive system, including ovary, uterus, placenta, and testis. Additionally, in *Xenopus*, *TPC3* appears to be enriched in ovary duct and ovary (transcript name—Xt7.1—CABI10995.3) (Gilchrist et al. 2004). Thus, TPC3-mediated Ca^{2+} signaling may play a role in reproduction. NAADP-induced Ca^{2+} signaling is important for fertilization of sea urchin eggs and starfish oocytes; however, its role in mammalian reproduction remains to be characterized (Guse and Lee 2008). Further investigation is needed to understand what signaling capacity was changed, which resulted in the degeneration of *TPC3* in the primate lineage leading to humans.

In summary, comparative genomics and evolutionary analysis of TPC3 represents our continuing efforts to determine the evolutionary systems biology (Medina 2005) of Ca^{2+} signaling pathways using large-scale genomic data. Our data provide the first evidence to demonstrate that an intracellular ion channel was relieved from selective pressure and became a pseudogene in the common ancestors of Apes and Old World monkeys. Together with TRPC2 studies (Liman and Innan 2003; Zhang and Webb 2003), our findings establish that the degeneration of two components of Ca^{2+} signaling at the plasma membrane and the intracellular stores occurred concomitantly at similar periods of primate evolution (fig. 5C) and thus broaden our current understanding of the regulation of ion channels and Ca^{2+} signaling in primate evolution.

Supplementary Material

Supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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