Paralogous murine *Nudt10* and *Nudt11* genes have differential expression patterns but encode identical proteins that are physiologically competent diphosphoinositol polyphosphate phosphohydrolases

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We previously described paralogous human genes {*NUDT10* and *NUDT11* [where NUDT is (nucleoside diphosphate attached moiety 'X')-type motif, also known as the 'nudix'-type motif]} encoding type 3 diphosphoinositol polyphosphate phosphohydrolases (DIPP3) [Hidaka, Caffrey, Hua, Zhang, Falck, Nickel, Carrel, Barnes and Shears (2002) J. Biol. Chem. **277**, 32730–32738]. Normally, gene duplication is redundant, and lacks biological significance. Is this true for the DIPP3 genes? We address this question by characterizing highly-conserved murine *Nudt10* and *Nudt11* homologues of the human genes. Thus these genes must have been duplicated prior to the divergence of primates and sciurognath rodents, approx. 115 million years ago, greatly exceeding the 4 million year half-life for inactivation of redundant paralogues; our data therefore indicate that the DIPP3 duplication is unusual in being physiologically significant. One possible functional consequence is gene neofunctionalization, but we exclude that, since *Nudt10* and *Nudt11* encode identical proteins. Another possibility is gene subfunctionalization, which we studied by conducting the first quantitative expression analysis of these genes. We demonstrated high *Nudt10* expression in liver, kidney and testis; *Nudt11* expression is primarily restricted to the brain. This differential, but complementary, expression pattern indicates that subfunctionalization is the evolutionary consequence of DIPP3 gene duplication. Our kinetic data argue that diphosphoinositol polyphosphates are more physiologically relevant substrates for DIPP3 than are either diadenosine hexaphosphate or 5-phosphoribosyl 1-pyrophosphate. Thus the significance of the *Nudt10*/*Nudt11* duplication is specific hydrolysis of diphosphoinositol polyphosphates in a tissue-dependent manner.

Key words: inositol pyrophosphates, type 3 diphosphoinositol polyphosphate phosphohydrolase (DIPP3).

INTRODUCTION

Turnover of the diphosphoinositol polyphosphates has been functionally linked to the control of homologous recombination [1], vesicle trafficking [2], the maintenance of cell-wall integrity [2], and the mediation of cellular responses to environmental stress [2]. The g5R gene in the African swine fever virus was recently found to encode an active diphosphoinositol polyphosphate phosphohydrolase (DIPP), suggesting a role for these compounds in viral morphogenesis [3]. Genetic manipulation of the turnover of diphosphoinositol polyphosphates in ovarian carcinoma cells affects apoptotic processes [4]. In order to account for such a diverse range of physiological processes being regulated by the turnover of diphosphoinositol polyphosphates, we [5,6] have suggested that metabolism of these molecules when they are bound to certain proteins acts as a molecular switch. This idea is akin to G-proteins that function as a binary switch between two interconvertible GTP-bound active and GDP-bound inactive states.

As diphosphoinositol polyphosphate turnover is so vital to cell function, it is important to characterize the enzymes that are responsible for this metabolism. We [7–9] have described five closely-related human nudix [(nucleoside diphosphate attached moiety 'X')-type motif, also known as NUDT] hydrolases [human (h)DIPPs types 1, 2α, 2β, 3α and 3β], which we have argued to preferentially hydrolyse diphosphoinositol polyphosphates. This proposal has not been universally accepted. For example, Bessman and colleagues have proposed that all of the nudix hydrolases specifically hydrolyse nucleoside diphosphates [10,11], which are completely metabolically unrelated to diphosphoinositol polyphosphates. A different group [12] recently proposed that the hDIPP3 enzymes 'do not contribute significantly' to the metabolism of diphosphoinositol polyphosphates *in vivo*, and instead they named these enzymes as diadenosine 5′,5‴-P^1,P^6-hexaphosphate (Ap_6A) hydrolases, reflecting what was viewed as being the more physiologically relevant substrates. More recently, a further function for the entire DIPP family was proposed, following the discovery that they can hydrolyse 5-phosphoribosyl 1-pyrophosphate (PRPP) to ribose 1,5 bisphosphate, a glycolytic activator [13]. One of the goals of the current study has been to clarify the physiological role of DIPP3 by examining the enzyme's substrate specificity.

Abbreviations used: Ap_6A, diadenosine 5′,5‴-P^1,P^6-hexaphosphate; DAPI, 4,6-diamidino-2-phenylindole; DIPP, diphosphoinositol polyphosphate phosphohydrolase (the human and murine forms are designated with a prefix of 'h' or 'm' respectively); DTT, dithiothreitol; EST, expressed sequence tag; FISH, fluorescence *in situ* hybridization; InsP_6, inositol hexakisphosphate; NTS, non-translated sequence; NUDT, (nucleoside diphosphate attached moiety 'X')-type motif, also known as the 'nudix'-type motif; *PP*-InsP_5, diphosphoinositol pentakisphosphate; [*PP*]$_2$-InsP_4, bisdiphosphoinositol tetrakisphosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY152853.

The hDIPP3α and hDIPP3β proteins are encoded by separate genes (*NUDT10* and *NUDT11*) [9]. We have proposed that these genes may have arisen by a gene duplication event [9], but it has not previously been shown whether or not this duplication might have any biological significance. Normally, this would not be the case. The most common repercussion of duplicated genes is redundancy, accompanied by a relatively rapid attrition of one of the paralogues, with a half-life of 4 million years [14]. Indeed, the maintenance of duplicated genes has the distinct disadvantage that they can serve as substrates for homologous recombination, with an ensuing loss or gain of genomic segments that results in human genetic disorders [15]. Only those duplicated genes that acquire functional significance are maintained for an extended period of time. Thus, one way to determine if a gene duplication has functional significance is to establish for how much evolutionary time the paralogues have been in existence and expressed. This was the approach we took in the present study.

There is considerable interest in the mechanisms by which a gene duplication might be maintained [16]. A recent model, known as 'duplication–degeneration–complementation' [17], argues that if a progenitor gene has a multifunctional state, these functions can be partitioned between daughter genes (a process known as subfunctionalization). A series of degenerative mutations is proposed to refine each gene to have a more restricted set of tasks, yet the two daughter genes are together proposed to complement the overall functions of the original gene. Although this hypothesis has been well received by population geneticists, clear-cut examples of its occurrence are still rare [18]. One way subfunctionalization is suggested to manifest itself is through differential patterns of expression of the two paralogues, that together recapitulate the original more complex expression characteristics of the progenitor gene [17]. To determine if this hypothesis might be applicable to DIPP3, we conducted the first quantitative analysis of the expression patterns of these genes.

EXPERIMENTAL

Materials

cDNA (accession number AB041576) encoding murine (m)DIPP3 was generously provided by Dr Katsuyuki Hashimoto (Division of Genetic Resources, National Institute of Infectious Diseases, Tokyo, Japan). ^{32}P-labelled dATP/dCTP and ^{3}H-labelled inositol hexakisphosphate (InsP_6) were purchased from NEN Life Science Products. ^{3}H-labelled Ap$_6$A, diphosphoinositol pentakisphosphate (*PP*-InsP_5) and bisdiphosphoinositol tetrakisphosphate $\{[PP]_2$-Ins$P_4\}$ were synthesized as described previously [9]. Recombinant hDIPP1 and its Glu^{66} $\rightarrow$ Gln inactive mutant were prepared as described previously [19]. PRPP was purchased from Sigma. All of the sequence alignments were obtained using GeneTool (version 1) and PepTool (version 2) with default settings (BioTools Inc., Edmonton, Canada).

DNA probe synthesis

All DNA probes used in this study were generated with a Prime-It® RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA, U.S.A.) or Strip-EZ™ DNA Kit (Ambion, Austin, TX, U.S.A.). Unincorporated nucleotides were removed by NucAway Spin Columns (Ambion).

Northern blotting

A 3′-non-translated sequence (NTS) 795 bp (nt 1089–1884) cDNA was obtained by digesting mDIPP3 cDNA (accession number AY152853) with *Dra*III and labelled with [^{32}P]dATP as probe. Another 3′-NTS 232 bp (nt 1274–1506) of XM_135786 was obtained by PCR amplification of FirstChoice™ PCR-Ready cDNA mouse testis (Ambion) with primers: 5′-ACTGGATCA-TCTGTCCTGTG-3′ (sense) and 5′-TCAAGGGAGTCGTGAG-ATAG-3′ (antisense). PCR conditions were: 94 °C for 3 min, followed by 35 cycles of: 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min; 72 °C for 10 min; and the PCR product was stored at 4 °C until analysis. The sequence of the PCR product was verified. Mouse RNA Master Blot (ClonTech, Palo Alto, CA, U.S.A.) was prehybridized for at least 30 min and hybridized in 10^6 cpm/ml of probe overnight at 42 °C in ULTRAhyb (Ambion). The blot was washed and developed as described in the Multiple Tissue Northern Blot manual (ClonTech). Blots were stripped in boiling 0.5 % SDS and re-probed for β-actin. Expression data were quantified with a Typhoon 8600 phosphoimager (Amersham Biosciences Corporation, Piscataway, NJ, U.S.A.), running the ImageQuant Software package (version 5.1).

Site-directed mutagenesis

A single nucleotide mutation of mDIPP3 cDNA was performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) with the following single mutation (underlined) oligonucleotides: 5′-GACTCGGTCAGCA<u>T</u>CGGCAGGAAGC-3′ (sense) and 5′-GCTTCCTGCCG<u>A</u>TGCTGACCGAGTC-3′ (antisense). This results in a cDNA which codes for mDIPP3, in which the naturally occurring Ile^{113} (see the Results section) replaces Thr^{113}. The sequence of the mutated cDNA was confirmed by sequencing with T3 and T7 primers, with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and a Prism 377 genetic analyser (Applied Biosystems).

Western-blot analysis

Mouse testis were frozen in liquid N_2, pulverized in a Tissumizer for 10 s, and extracted in buffer containing 50 mM β-glycerophosphate (pH 8.2), 250 mM sucrose, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 4 mM CHAPS, 250 μM 4-(2-aminoethyl)benzene-sulphonylfluoride, 10 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 1 μg/ml pepstatin and 1 μg/ml leupeptin. Samples were centrifuged at 18 000 ***g*** for 30 min at 4 °C, and the supernatants were stored at − 70 °C. Anti-hDIPP3 antibodies were then employed for Western-blot analysis as described previously [9].

Expression and purification of mDIPP3 protein

The entire coding region of the cDNA AB041576, with the single nucleotide mutation described above, was amplified by PCR using a 5′ primer containing a *Bam*HI site, and a 3′ primer containing a *Sal*I site. The resultant 495 bp PCR product was digested with *Bam*HI and *Sal*I, purified from the agarose-gel, and ligated into the cognate sites of vector pQE30 (Qiagen, Valencia, CA, U.S.A.). This strategy generates a recombinant protein containing the amino acid sequence MRGSHHHHHHGS upstream of the complete mDIPP3 coding region. The recombinant polyhistidine-tagged mDIPP3 was expressed in *Epicurian coli* BL21 competent cells (Stratagene), and expression was induced with 1.0 mM isopropyl β-D-thiogalactoside for 4–5 h at 30 °C. Cells were harvested by centrifugation, and the enzyme was affinity purified

by Ni^{2+}-agarose FPLC chromatography as described previously [20].

Enzyme assays

Assays of substrate hydrolysis by recombinant DIPP were performed as follows, unless otherwise stated: hydrolysis of ^{3}H-labelled diphosphoinositol polyphosphates was assessed at 37 °C in buffer containing 50 mM KCl, 25 mM Hepes (pH 7.2 with KOH), 2 mM DTT, 2 mM $MgSO_4$, 1 mM Na_2EDTA and 0.1 mg/ml BSA. The reaction rates were determined after HPLC of substrates and products, as described previously [9]. Hydrolysis of [^{3}H]Ap_6A was assayed in buffer containing 50 mM Hepes (pH 7.6 with NaOH), 2 mM DTT, 1 mM $MnCl_2$ and 0.1 mg/ml BSA. Reaction rates were determined as described previously [21]. Following PRPP hydrolysis in buffer containing 25 mM Tris/HCl (pH 8.5), 5 mM magnesium gluconate, 1 mM DTT and 0.1 mg/ml BSA, the P_i released was subsequently assayed as described previously [22].

Fluorescence *in situ* hybridization (FISH) mapping

The 2.4 kb mDIPP3 cDNA (accession number AY152853) was biotinylated with dATP using Gibco BRL BioNick labelling kit (15 °C for 1 h) [23]. Human lymphocyte chromosomal spreads were prepared as described previously [23], and labelled probes were hybridized overnight in a hybridization buffer containing 50% formamide and 10% dextran sulphate. After washing, FISH signals and 4,6-diamidino-2-phenylindole (DAPI) banding pattern were recorded separately [23]. Images were captured and combined by charge-coupled-device camera, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes [23].

RESULTS

Identification and analysis of two mDIPP3 genes on chromosome X

When a gene-duplication event is redundant, one of the paralogous genes is silenced with a half-life of 4 million years [14]. An important goal of the present study was to obtain information concerning the evolutionary age of the gene duplication event that gave rise to the two human DIPP3 genes. We therefore searched for evidence that a similarly structured pair of DIPP3 genes might occur in other genomes. BLAST searches revealed GenBank® database entries that are candidate murine homologues of the hDIPP3 mRNAs. We obtained one of these cDNAs [a Riken expressed sequence tag (EST) cDNA, with the accession number AB041576], and its sequence was confirmed (see the Experimental section). However, we noted that, according to the annotation that accompanies AB041576, the final 8 nt of the 3′-terminus do not represent part of the original mRNA, but instead comprise engineered restriction sites. These 8 nt are not present in the sequence of the cDNA which we have deposited into the GenBank® database (accession number AY152853). Instead, our 2.4 kb sequence terminates with a polyadenylated tail beginning 15 nt downstream of a canonical AATAAA polyadenylation signal. The nucleotide sequences of AY152853 appears to be homologous to the cDNA for hDIPP3β; in particular, the coding regions are 88% identical and the 3′-NTSs are approx. 80% identical (Figure 1). The sequence of the murine gene (which has been named *Nudt11*) is contained in a genomic contig (NW_042618, Figure 2). This contig also contains sequence that suggests the existence of a second murine DIPP3 gene, which has been named *Nudt10* (Figure 2). From the *Nudt10* genomic sequence, and a number of matching ESTs, the GenBank database has created a 'model' 1.6 kb cDNA, XM_135786. By joining this model sequence to an additional, overlapping EST cDNA (AI853080), we have extended it to a 1.9 kb contig that terminates in a polyadenylated tail (Figures 1 and 2).

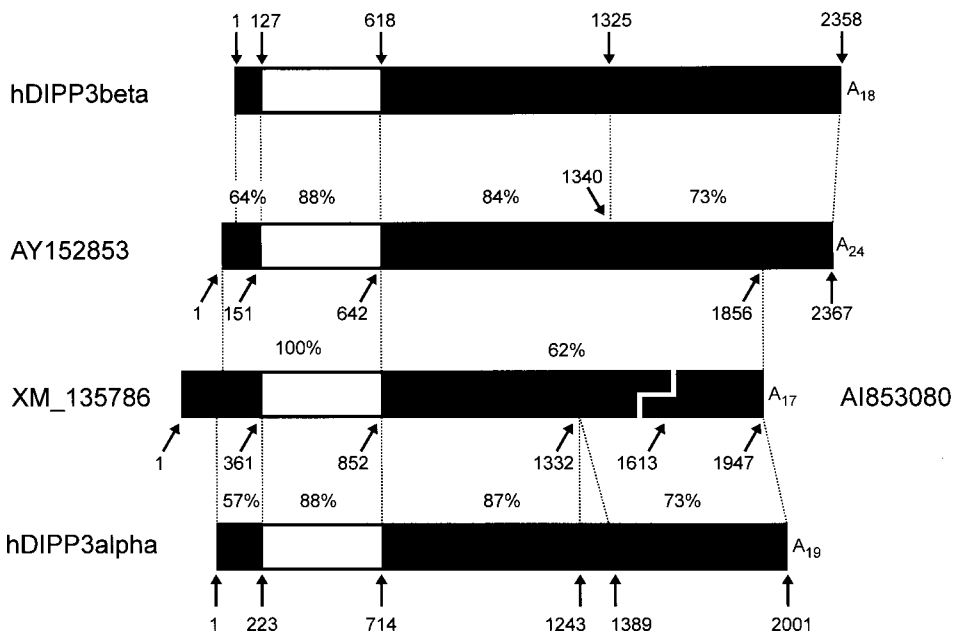

Figure 1 Alignment of murine and human DIPP3 nucleotide sequences

The Figure shows the alignment of four nucleotide sequences: the hDIPP3β cDNA (accession number BC009942); a mDIPP3 cDNA (accession number AY152853); a contiguous sequence that combines a 'model' murine cDNA (accession number XM_135786) with an overlapping EST cDNA (accession number AI853080) (the extent of the overlap, 125 nt, is illustrated by the 'break'); and the hDIPP3α cDNA (EST AF469196). Nucleotide numbers for each cDNA are also indicated. The coding regions are shown in white; NTSs are black. Also, the percentage identities of regions demarcated by vertical broken lines are shown.

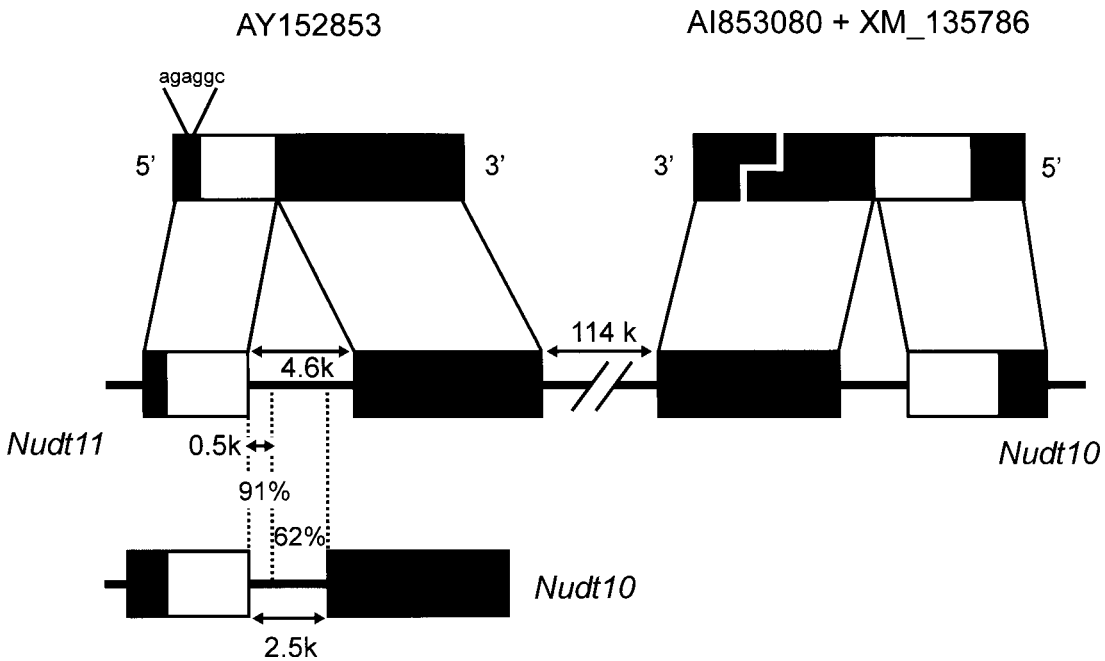

Figure 2 Alignment of murine cDNAs with corresponding genomic sequence

The Figure depicts an alignment of a mouse genomic contig (accession number NW_042618) with the corresponding mouse cDNAs. The combined sequence (AI853080 + XM_135786) is > 99 % identical to the corresponding exonic genomic sequence. The cDNA AY152853 has a 6 nt insertion (shown in the Figure) and 12 single nt differences compared with the corresponding exonic genomic sequence, including three in the 5′-NTS, one in the coding region and eight in the 5′-NTS. The percentage identities of the proposed introns are also indicated. The assignment of *Nudt10* and *Nudt11* as gene names reflects the sequence homology with their human orthologues. This nomenclature has been approved by the Mouse Genome Nomenclature Committee.

The putative transcript of the *Nudt10* gene shares virtually identical coding and 5′-NTS regions with the proposed *Nudt11* transcript (Figure 1). However, the 3′-NTSs are rather different (62 % identity, Figure 1). Interestingly, the *Nudt10* transcript is homologous to the cDNA for hDIPP3α (Figure 1). This is an important point: the 3′-NTSs of the two putative murine cDNAs are less similar in sequence to each other (62 % identical) than are their individual similarities to their corresponding human homologues (73–87 %; see Figure 1). Thus there appear to be murine homologues of both the type α and β hDIPP3 genes (i.e. *NUDT10* and *NUDT11*).

The genomic contig (NW_042618) predicts that the *Nudt10* and *Nudt11* genes encode identical proteins. However, alignment of our *Nudt11* cDNA with the corresponding genomic sequence (Figure 2) revealed a single nt inconsistency within the coding region; nt 488 in our *Nudt11* cDNA sequence (AY152853) is C but this aligns with T in the *Nudt11* genomic sequence. This has an impact upon the amino acid sequence of the predicted *Nudt11* protein, changing Thr^{113} (encoded according to the cDNA) to Ile (encoded according to genomic sequence, see Figure 3). In order to adjudicate this nt difference, we screened the GenBank® database for EST sequences that overlapped this nucleotide, yet could also be distinguished as being transcripts of *Nudt11* and not *Nudt10* (which we determined by comparing 3′-NTS sequence). We found seven *Nudt11* ESTs that met this criteria: AA087425 (lung); AA388051 (embryo); AA388147 (embryo); BB628784 (brain); BB664803 (eyeball); BM948161 (brain); BM950387 (brain). All of these *Nudt11* ESTs contain T at the appropriate position, in agreement with the genomic sequence. We propose that it is likely that a PCR amplification error was introduced C into the *Nudt11* cDNA when the clone (AB041576) was initially constructed, although we cannot exclude the alternate possibility of there being a rare nucleotide polymorphism.

Figure 3 Amino acid sequences of hDIPP3β and mDIPP3

The accession number for hDIPP3β is BC009942. The protein sequence for mDIPP3 that is shown is verified by the following nt sequences (accession numbers): XM_135786, AA087425, AA388051, AA388147, BB628784, BB664803, BM948161 and BM950387. We have also deposited a mDIPP3 cDNA sequence into GenBank® database (accession number AY152853), but it is likely that this particular cDNA has a single nt PCR error, that encodes threonine at position 113 instead of isoleucine (see the text for details).

Nevertheless, we can also conclude that, in most cases at least, the *Nudt10* and *Nudt11* genes encode an identical protein with isoleucine at position 113 in the amino acid sequence (Figure 3). Indeed, the isoleucine residue is also conserved in hDIPP3 (Figure 3), as well as in hDIPP1 and hDIPP2 [8]. Multiple sequence alignments indicate that the mDIPP3 is more similar to hDIPP3β (96 % identical, Figure 3), than to either hDIPP2 (87 % identical, results not shown) or hDIPP1 (75 % identical, results not shown).

In addition to the exonic similarities between the murine *Nudt10* and *Nudt11* genes, they each have a predicted intron which also shows considerable sequence similarity (Figure 2). The two proposed genes are encoded on NW_042618, just

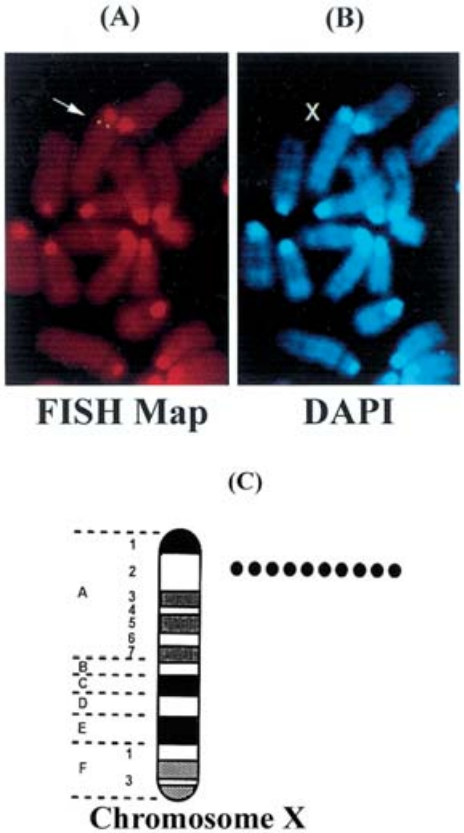

Figure 4 Chromosomal localization of mDIPP3 by FISH

(**A**) FISH signals (yellow dots indicated by the arrow). (**B**) The same spread, as seen in (**A**), stained with DAPI to identify chromosomes. Chromosome X is marked. (**C**) An ideogram of the DAPI banding pattern on the relevant section of chromosome X. Each dot represents the FISH signals detected in the regions XA2.

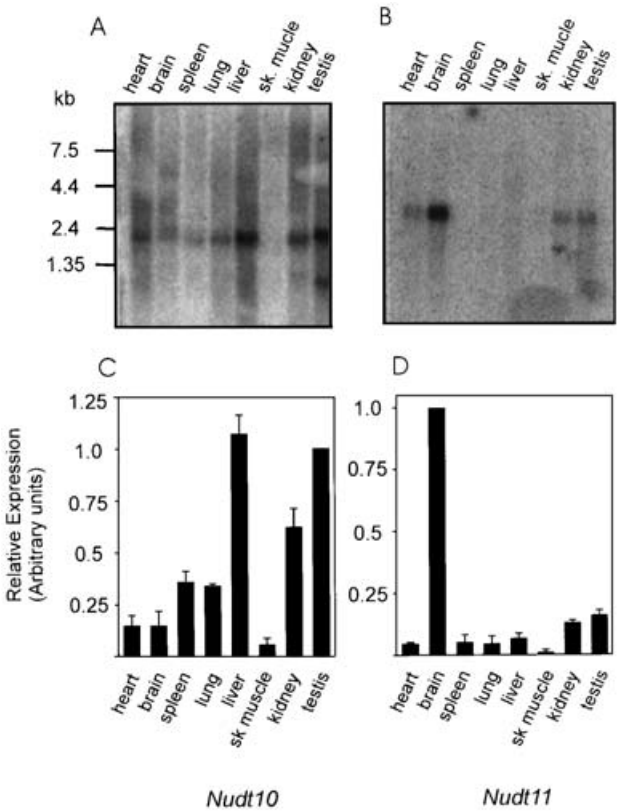

Figure 5 Northern analysis of mDIPP3 mRNA

A mouse Multiple Tissue Northern blot (ClonTech) was probed with 795 bp (nt 1089–1884) 3′-NTS of *Nudt11* transcript (**B**). The blot was stripped in boiling 0.5 % SDS, and then reprobed with a 232 bp (nt 1274–1506) 3′-NTS of the *Nudt10* transcript (**A**). The blot was then stripped a second time and probing with a human β-actin probe (results not shown). Band intensities were quantified (see the Experimental section) relative to the β-actin signal in each lane, and the data (mean values from two blots $\pm$ range) are normalized to band intensity in either testis (**C**) or (**D**).

114 kb apart (Figure 2). This unusual convergently transcribed gene arrangement mirrors that of the two human *NUDT10* and *NUDT11* genes [9]. An intron/exon boundary that we identified to occur at the junction of the coding region and 3′-NTS in both murine genes (Figure 2) is also conserved in the human genes [9]. We have mapped these mouse *Nudt* genes to chromosome X by FISH (Figure 4). The corresponding human genes have also been mapped to chromosome X [9]. This conservation of the genomic architecture in the two species indicates that a single DIPP3 gene-duplication event has taken place, prior to the divergence of primates and sciurognath rodents, which occurred approx. 115 million years ago [24].

Expression of mDIPP3

To further investigate if the mDIPP3 gene duplication is functionally significant, we next investigated whether or not both *Nudt10* and *Nudt11* are expressed. We first prepared PCR primers that were specific for the 3′-NTS of the *Nudt10* gene. We generated a 232 bp PCR product by amplification of a mouse testis cDNA library (see the Experimental section). The product was sequenced and found to match the sequence of the *Nudt10* gene. Using this PCR amplification product as a probe for Northern analysis, we found that an *Nudt10* mRNA of approx. 2 kb was prominently expressed in testis, liver and kidney, with weaker expression in all other tissues tested, including the brain (Figures 5A and 5C). The size of this mRNA is close to the 1.9 kb that we predicted for this transcript (Figure 1). The high levels of expression of *Nudt10* in liver and kidney contrast with the low level of expression of either of the two human DIPP3 transcripts in these particular tissues (see [9]).

We next prepared a 795 bp probe that was specific for the 3′-NTS of the *Nudt11* gene. Northern analysis of a multiple murine tissue blot revealed that a mRNA of approx. 3.5 kb was primarily expressed in the brain, with relatively weak expression in all other tissues that were examined (Figures 5B and 5D). Thus we conclude that both mouse *Nudt10* and *Nudt11* genes are expressed, but in a differential and complementary manner. The distinction in the pattern of expression of the *Nudt10* and *Nudt11* genes is the first observation that there is a significant, quantitative difference between these two gene products. This phenomenon could represent subfunctionalization of duplicated genes, in the manner predicted by the duplication–degeneration–complementation hypothesis [17].

We also probed a mouse testis homogenate with anti-peptide antibodies originally raised against the C-terminus of hDIPP3 [9]. We detected a 23 kDa protein (Figure 6), which corresponds to the size of the hDIPP3 protein (Figure 6 and [9]). There was not a corresponding signal when pre-immune serum was used (Figure 6). These results confirm that the mDIPP3 protein is expressed.

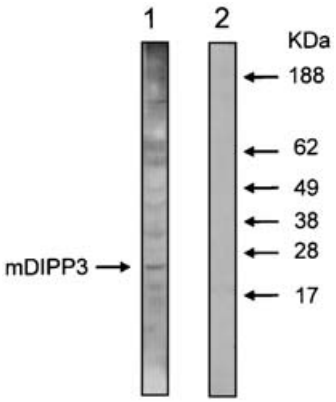

Figure 6 Detection of mouse DIPP3 protein by Western-blot analysis

Extracts (150 μg protein) of mouse testis were subjected to Western-blot analysis (see the Experimental section) with either hDIPP3 antisera (500-fold dilution, lane 1) or pre-immune serum (lane 2).

DTT and DIPP3 activity

In a recent study of the two hDIPP3 enzymes [9], we established that the values of the specificity constants (k_{cat}/K_m) for *PP*-InsP_5 hydrolysis were 18 and 42 $M^{-1} \cdot s^{-1} \times 10^5$ for the α and β isoforms respectively. In that previous study [9], the hDIPP3 proteins were purified and assayed in medium containing 2 mM DTT. When we then omitted DTT, we could not detect any hydrolysis of $[PP]_2$-InsP_4 by recombinant hDIPP3 (results not shown). Furthermore, the omission of DTT reduced by 2 orders of magnitude our values for the specificity constant for the hydrolysis of *PP*-InsP_5 to 0.026 and 0.127 $M^{-1} \cdot s^{-1} \times 10^5$ for the α and β isoforms respectively. These results raise the possiblity that, *in vivo*, changes in the redox environment in the vicinity of the hDIPP3 proteins might regulate their catalytic activity towards diphosphoinositol polyphosphates. Nevertheless, our results suggest that the potential ability of the hDIPP3 enzymes to hydrolyse diphosphoinositol polyphosphates would be underestimated if the proteins were not to be purified and assayed in the presence of DTT.

Catalytic activity of mDIPP3 towards diphosphoinositol polyphosphates

We have described above our evidence that two murine genes (*Nudt10* and *Nudt11*) encode an identical DIPP3 protein. The mDIPP3 protein (Figure 3) was expressed in *E. coli* with a polyhistidine tag and was subsequently purified (Figure 7). The purification and assay of mDIPP3 was conducted in the presence of 2 mM DTT (see above). The purified recombinant protein migrated on SDS/PAGE with an apparent size of 25 kDa (Figure 7A), since, due to the N-terminal polyhistidine tag, it is a little larger than the native protein (Figure 6). We then incubated the recombinant protein with *PP*-[^{3}H]InsP_5. HPLC analysis (Figure 7B) demonstrated that the mDIPP3 specifically removed the β-phosphate from the diphosphate group of *PP*-InsP_5, yielding InsP_6. When the recombinant mDIPP3 was incubated with $[PP]_2$-[^{3}H]InsP_4, little or no *PP*-InsP_5 accumulated, as this itself was rapidly dephosphorylated to InsP_6 (Figure 7C). The specificity constants (k_{cat}/K_m) for *PP*-InsP_5 and $[PP]_2$-InsP_4 have values that do not substantially differ from those of its human homologue, namely, hDIPP3β (Table 1). These results illustrate that the catalytic activity of DIPP3 is well conserved between humans and mice.

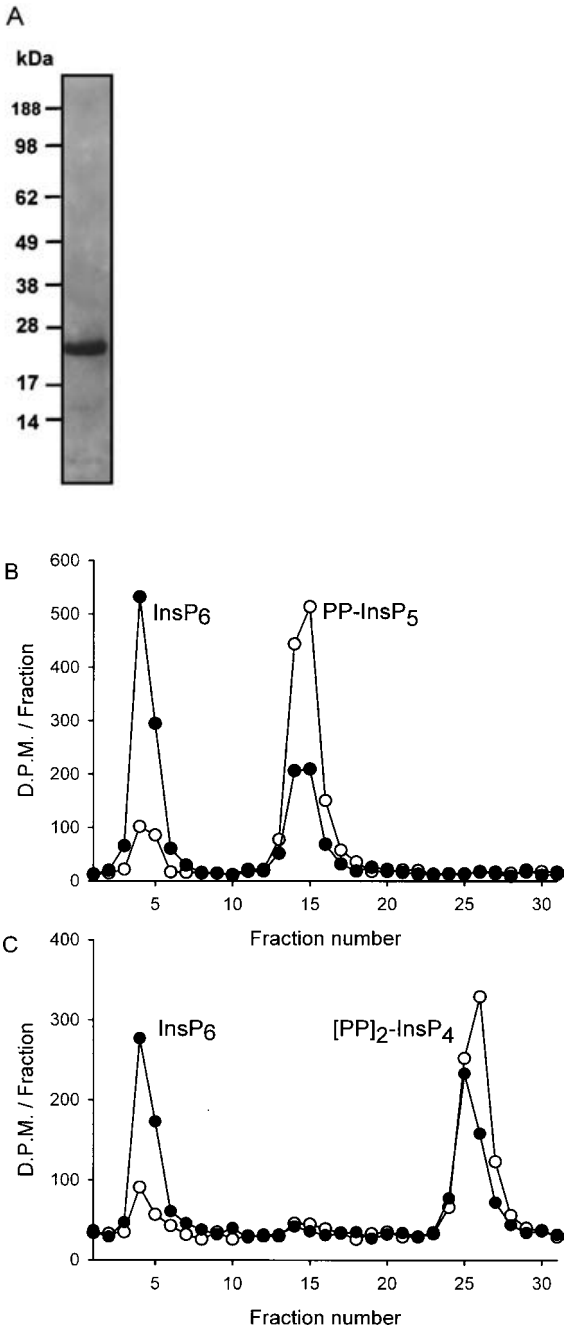

Figure 7 Expression of recombinant mDIPP3

(**A**) Purified recombinant mDIPP3 (3 μg) was resolved on a 4–12 % NuPage® Bis-Tris gel in Mes buffer, according to the manufacturer's instructions, and then stained with Coomassie Blue. (**B**) Recombinant mDIPP3 (0.25 ng) was incubated at 37 °C (●) or on ice (○) for 15 min with 1300 dpm *PP*-[^{3}H]InsP_5 in 100 μl of assay buffer (see the Experimental section). The reaction mixtures were quenched and analysed by HPLC. (**C**) Recombinant mDIPP3 (4 ng) was incubated at 37 ° (●) or on ice (○) for 45 min with 900 dpm $[PP]_2$-[^{3}H]InsP_4 in 4300 μl of assay buffer (see the Experimental section). The reaction mixtures were quenched and analysed by HPLC.

Catalytic activity of mDIPP3 towards Ap_6A

The DIPP family can hydrolyse diadenosine polyphosphates, such as Ap_6A, at least *in vitro* [8,12,25]. It was previously been noted that the optimum rate of hydrolysis of Ap_6A by recombinant hDIPP3 *in vitro* requires millimolar concentrations of Mn^{2+} [12]. In just such assay conditions, we obtained a specificity constant

Table 1 Catalytic properties of mDIPP3 and hDIPP3

The specificity constants for hydrolysis of Ap_6A, *PP*-InsP_5 and $[PP]_2$-InsP_4 were determined as described in the Experimental section. Results are expressed as the mean ± S.E.M. ($n=3$). The results for hDIPP3β are taken from [9].

	k_{cat}/K_m ($10^{-5} \times M^{-1} \cdot s^{-1}$)	
Substrate	mDIPP3	hDIPP3β
Ap_6A	0.073 ± 0.006	0.22
PP-InsP_5	10.8 ± 2	42
$[PP]_2$-InsP_4	4.1 ± 0.7	4.9

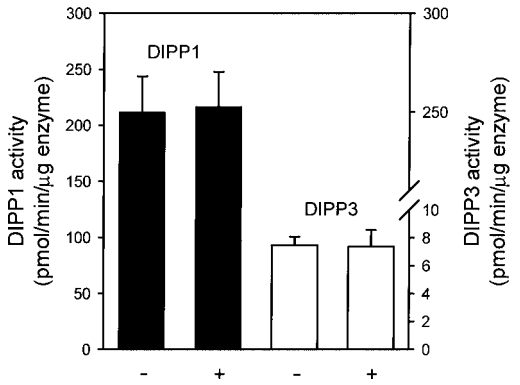

Figure 8 Lack of effect of PRPP upon DIPP activity

Either hDIPP (0.47 μg; ■) or mDIPP3 (8.4 μg; □) was incubated (as described in the Experimental section) with 0.08 μM *PP*-[^{3}H]InsP_5 for 30 min in either the presence (+) or absence (−) of 1 mM PRPP. Assays were analysed by HPLC (see the Experimental section). Results are expressed as the means ± S.E.M. ($n=3$). Note the break in the scale on the right-hand *y*-axis.

for hydrolysis of Ap_6A by mDIPP3 that was 50–150 less than the corresponding values for hydrolysis of diphosphoinositol polyphosphates (Table 1). Moreover, it is acknowledged that the physiologically relevant concentration of Mn^{2+} is <0.5 μM [12]. We could not detect any Ap_6A hydrolysis by mDIPP3 when the enzyme was incubated with 0.5 μM Mn^{2+} in the physiologically relevant buffer (1 mM Mg^{2+}; see the Experimental section) that was used to record dephosphorylation of *PP*-InsP_5 and $[PP]_2$-InsP_4 (results not shown). So even under *in vitro* assay conditions that probably over-estimate the rate of hydrolysis of Ap_6A by DIPP *in vivo*, we have determined that diphosphoinositol polyphosphates are preferred as substrates over Ap_6A (Table 1).

To what extent is PRPP a substrate for DIPPs?

A recent report [13] proposed that the DIPP family have an additional function as hydrolases that convert PRPP to ribose 1,5 bisphosphate, a glycolytic activator. These authors [13] determined that hDIPP1 and hDIPP3 hydrolysed PRPP at pH 8.5 in medium containing 5 mM Mg^{2+}. We confirmed that hDIPP1 did indeed hydrolyse 100 μM PRPP under these conditions; the activity that we observed (2.8 μmol of PRPP hydrolysed/min per μmol of hDIPP1) agrees well with the rate of 3.9 μmol of PRPP hydrolysed/min per μmol of hDIPP1 that was observed by Fisher et al. [13]. We further showed that this hydrolysis was not catalysed by a contaminant in the preparations of recombinant enzyme, as we could not detect any hydrolysis of PRPP by a catalytically inactive $Glu^{66} \rightarrow Gln$ mutant of hDIPP1 (results not shown). However, when hDIPP1 was incubated in the same medium that we used to assay hydrolysis of diphosphoinositol polyphosphates (see above), which has a physiological pH of 7.2, the rate of PRPP hydrolysis was more than 60-fold slower (0.06 μmol of PRPP hydrolysed/min per μmol of hDIPP1) compared with the rates we obtained at pH 8.5. We therefore designed assays to investigate to what extent PRPP may compete with *PP*-InsP_5 *in vivo*. The rate of hDIPP1-mediated hydrolysis of 0.08 μM *PP*-InsP_5 (>10-fold below physiological levels, see [13]) was 212 ± 32 pmol/min per μg of enzyme (Figure 8). PRPP levels in mammalian cells are below 10 μM [26], yet even 1 mM PRPP (100-fold above physiological levels), did not significantly affect the rate of hydrolysis of 0.08 μM *PP*-InsP_5 by hDIPP1 (Figure 8). We further noted that mDIPP3 was 30-fold less active towards *PP*-InsP_5 than was hDIPP1 (Figure 8), but even so, 1 mM PRPP still failed to significantly compete with *PP*-InsP_5 for hydrolysis by mDIPP3 (Figure 8). We therefore conclude that under conditions where DIPP1 and DIPP3 have free access to *PP*-InsP_5 and PRPP, the latter will not be a physiologically relevant substrate. In the absence of any evidence that DIPP3 and DIPP1 have different substrate preferences, we propose that it is their distinct catalytic efficiencies that is the functionally important factor that distinguishes these two phosphohydrolases.

DISCUSSION

The discovery of an apparently duplicated pair of human DIPP3 genes [9,12] was not previously known to have any functional significance. Indeed, the most frequent outcome of gene duplication is redundancy, such that one paralogue becomes inactivated [14]. In contrast, in the present study we demonstrate that the DIPP3 gene duplication has been a functionally significant event in the evolution of the mammalian genome. We also show how the existence of the DIPP3 gene pair fits with contrasting theories [17] on the mechanisms by which certain paralagous genes can be maintained.

We have also studied DIPP3 substrate specificity. Previous work has shown that the DIPP family will hydrolyse Ap_6A [12,25]. However, hydrolysis of Ap_6A by hDIPP3 is optimized by millimolar levels of Mn^{2+} (Table 1 and [12]), which exceeds physiological Mn^{2+} concentration (<0.5 μM [12]). In addition, the specificity constants for hydrolysis of Ap_6A by hDIPP3 and mDIPP3, in medium containing millimolar levels of Mn^{2+}, are still 50–150-fold less than the specificity constants for Mg^{2+}-dependent *PP*-InsP_5/$[PP]_2$-InsP_4 hydrolysis (Table 1). Of course, conclusions concerning an enzyme's relative activity towards alternative substrates do not rely solely upon the specificity constants, but must also take into account the concentrations of these competing metabolites [27]. Recent results have led to the expectation that mammalian cell cytosol probably contains only low nanomolar levels of these diadenosine polyphosphates [13,28]. In contrast, levels of *PP*-InsP_5 and $[PP]_2$-InsP_4 in cells are routinely estimated to be in the 1 μM range (see [13]). This consideration of relative substrate levels further supports our conclusion that the role of hDIPP3 and mDIPP3 *in vivo* is to hydrolyse diphosphoinositol polyphosphates, and not diadenosine polyphosphates. The fact that DIPP3 has lower catalytic efficiency than DIPP1 and DIPP2 [9,12] implies that tissue- and developmentally specific expression of the various DIPP isoforms provides a mechanism for regulating the extent of cellular turnover of diphosphoinositol polyphosphates.

Another potential additional function for the DIPP family was recently suggested by the discovery [13] that, *in vitro*, these

enzymes also hydrolyse PRPP to ribose 1,5-bisphosphate, a glycolytic activator. We have now confirmed that, in assays where PRPP is the only available substrate, it can indeed be hydrolysed by DIPP1, particularly when the pH is 8.5. However, we noted that enzyme activity was 60-fold slower under physiologically relevant conditions at pH 7.2 (see the Results section). Moreover, we directly studied the ability of PRPP to compete with *PP*-InsP_5 for hydrolysis by DIPP *in vitro*. We even used assay conditions that were designed to favour PRPP hydrolysis; the concentration of PRPP that was chosen (1 mM) is up to 100-fold in excess of physiological levels. The concentration of *PP*-InsP_5 (0.08 μM) is >10-fold less than levels *in vivo*. Even so, PRPP did not significantly affect the rate of *PP*-InsP_5 hydrolysis by either hDIPP1 or mDIPP3 (Figure 8).

We have also shown that the genomic architecture of the human DIPP3 genes is remarkably well conserved in the mouse genome (Figures 1 and 2). This conservation applies not just to the gene sequences, but also to the intron/exon boundaries, their proximity to each other on chromosome X, and their unusual convergently transcribed gene orientation. The importance of this observation is that it establishes that a single DIPP3 duplication event has occurred, prior to the divergence of primates and sciurognath rodents, 115 million years ago [24]. Duplicated genes are typically redundant, and in such a situation, one duplicate is inactivated with a half-life of only 4 million years [14]. Indeed, duplicated genes are potentially hazardous, because they offer a substrate for homologous recombination and the accompanying loss or gain of genomic information [15]. The DIPP3 genes could only be maintained in the genome for over 115 million years if their duplication has considerable functional significance. This is one of the important conclusions to arise from our work.

We have considered whether the expression of two DIPP3 genes has been maintained by their acquisition of separate catalytic activities (i.e. 'neofunctionalization', see [17]). Previous studies of the human DIPP3 proteins do raise this as a possibility, since they are slightly different in sequence [9,12]. In contrast, we show in the present study that the two mDIPP3 genes in mice can encode identical proteins. This observation indicates that the evolutionary pressure to maintain two hDIPP3 genes is independent of their single amino acid difference. Thus, neofunctionalization does not appear to explain the maintenance of the DIPP3 paralogues.

A new paradigm to explain both the preservation and the significance of duplicated genes has recently emerged. This model, known as duplication–degeneration–complementation [17], argues that if a progenitor gene has a multifunctional state, these functions can be partitioned between daughter genes (a process known as subfunctionalization; [17]). A series of degenerative mutations is proposed to refine each gene to have a more restricted set of tasks, yet the two daughter genes are proposed to complement the overall functions of the original gene. Although this hypothesis has been well received by population geneticists, clear-cut examples of its occurrence are still rare [18]. One way subfunctionalization is suggested to manifest itself is through differential patterns of expression of the two paralogues, that together complement the original expression pattern of the progenitor gene [17]. We provide new support for this idea in the present study. We have shown that the murine *Nudt10* and *Nudt11* genes are expressed in a differential and complementary manner (Figure 5). The existence of paralagous DIPP3 genes therefore adds to the repertoire of mechanisms regulating tissue-specific rates of diphosphoinositol polyphosphate turnover, presumably through variability in gene expression control elements. This improves flexibility in the response times for the molecular switching activity which we [5,6] have proposed underlies the ongoing turnover of the diphosphoinositol polyphosphates.

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