Diphthamide modification of eEF2 requires a J-domain protein and is essential for normal development

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Summary
The intracellular target of diphtheria toxin is a modified histidine residue, diphthamide, in the translation elongation factor, eEF2 (also known as EFT1). This enigmatic modification occurs in all eukaryotes and is produced in yeast by the action of five gene products, DPH1 to DPH5. Sequence homologues of these genes are present in all sequenced eukaryotic genomes and, in higher eukaryotes, there is functional evidence for DPH1, DPH2, DPH3 and DPH5 acting in diphthamide biosynthesis. We identified a mouse that was mutant for the remaining gene, Dph4. Cells derived from homozygous mutant embryos lacked the diphthamide modification of eEF2 and were resistant to killing by diphtheria toxin. Reporter-tagged DPH4 protein localized to the cytoskeleton, in contrast to the localization of DPH1 and consistent with evidence that DPH4 is not part of a proposed complex containing DPH1, DPH2 and DPH3. Mice that were homozygous for the mutation were retarded in growth and development, and almost always die before birth. Those that survive long enough had preaxial polydactyly, a duplication of digit 1 of the hind foot. This same defect has been seen in embryos that were homozygous for mutation of DPH1, suggesting that lack of diphthamide on eEF2 could result in translational failure of specific proteins, rather than a generalized translation downregulation.

Key words: RNA translation, Diphthamide, Mouse mutation

Introduction
Diphthamide is an enigmatic posttranslational modification occurring on a single amino acid residue of a single protein in the eukaryotic proteome. Diphthamide modification is present in all eukaryotic organisms, in which it is restricted to a histidine residue of translation elongation factor 2 (eEF2, also known as EFT1; position 715 in mammals and 699 in yeast) (Robinson et al., 1974; van Nesset et al., 1980). There is also evidence of a diphthamide modification, or a precursor of diphthamide, in the analogous elongation factor of archaeabacteria (Pappenheimer et al., 1983). However, it is absent from EF-G, the eubacterial orthologue of eEF2. The modification produces a lethal blockage in protein synthesis (reviewed by Collier, 2001).

The normal function of diphthamide is not entirely clear. Within the eEF2 structure, the modified histidine is located at the tip of a domain loop that is proposed to mimic the anticodon loop of tRNA (Jorgenson et al., 2005; Jorgenson et al., 2006; Ortiz et al., 2006). It has been suggested that this loop, bearing the diphthamide modification, stabilizes the tRNA-anticodon–mRNA-codon interaction and is necessary to maintain the translation reading frame. Analysis of cells lacking diphthamide shows that unmodified eEF2 results in an increase of –1 frameshifting during translation (Ortiz et al., 2006).

Diphthamide biosynthesis has been well characterized in yeast and has been shown genetically to require the action of five proteins, DPH1–DPH5 (Chen et al., 1985; Liu et al., 2004). Functional homologues of four of these – DPH1, DPH2, DPH3 and DPH5 – have been identified in mammals, by identification of mutant CHO cells, in which they have been called, respectively, CG-4, CG-3, CG-2 and CG-1 (Liu and Leppla, 2003; Moehring and Moehring, 1979; Moehring et al., 1980; Moehring et al., 1984; Nobukuni et al., 2005). Diphthamide-deficient yeast strains have allowed analysis of the pathway, which proved to be a complex, multi-step process (Liu et al., 2004). The first step involves the transfer of a 3-amino-3-carboxypropyl moiety from S-adenosylmethionine (AdoMet) to the C-2 imidazole of the target histidine to produce an intermediate, which proved to be a complex, multi-step process (Ortiz et al., 2005). Diphthamide-deficient yeast strains have allowed analysis of the pathway, which proved to be a complex, multi-step process (Liu et al., 2004). The first step involves the transfer of a 3-amino-3-carboxypropyl moiety from S-adenosylmethionine (AdoMet) to the C-2 imidazole of the target histidine to produce an intermediate, which proved to be a complex, multi-step process (Liu et al., 2004). The final step is the amidation of the side-chain carboxyl group of diphthine by an ATP-dependent enzyme, resulting in diphthamide. No mutations have been identified in this final step because diphthine can also be ADP-ribosylated by DT and ETA, although at a slower rate than is diphthamide (Moehring and Moehring, 1979), meaning that genetic selection cannot be used, and an ATP-dependent amidation enzyme is still to be identified (Liu et al., 2004).

The yeast Dph4 gene has an orthologue that is detectable in mammalian genome sequences, but no functional data is available. Furthermore, no mutations corresponding to Dph4 have been found in CHO cells. Here, we describe the identification of a mutation in the mouse orthologue of Dph4, from a novel genetic screen for lethal mutations. The protein product of Dph4 contains a J-domain, which is the hallmark of the Dnaj or Hsp40 protein family, suggesting that it acts as an Hsp70 co-chaperone. Homozygous mutation of Dph4 results in the absence of diphthamide on eEF2
in embryos and resistance to DT in mutant cells derived from them. The homozygous mutation is generally lethal by embryonic day 14 (E14), and embryos have retarded growth from as early as E9. However, if the homozygous embryos survive long enough to begin digit formation, the majority have a distinctive polydactyly phenotype on their hind limbs, similar to embryos that are mutant in Dph1 (Chen and Behringer, 2004).

Results
A genetic screen for lethal mutations on mouse chromosome 2
We established a genetic screen in mice to identify both visible and lethal mutations in the vicinity of the Pax6 gene (chromosome 2). The screen is illustrated in Fig. 1A. In brief, mutagenized male mice are crossed with females carrying a point mutation in Pax6, which confers a small-eye phenotype (Pax6Sey-Neu) (Hill et al., 1991). Small-eyed offspring from this cross carry a potentially mutant chromosome 2, opposite the mutant Pax6, and these are crossed again to mice bearing a deletion (Pax6Sey-1H) (Kent et al., 1997) encompassing Pax6 and other neighboring genes (Fig. 1B). Offspring from this cross have four potential genotypes; one class, with the compound Pax6Sey-Neu/Sey-1H genotype, died around birth. Two classes of genotype had small eyes: those carrying either the point mutation or the deletion. The latter animals are phenotypically distinct from the point-mutation carriers by virtue of their smaller size and unpigmented spots on the belly and feet, and these animals will potentially have a newly induced point mutation in one of the genes spanned by the deletion. If this new mutation has a recessive phenotype, it will be revealed by the deletion and observed in these mice. If the phenotype is lethal, the deletion class will be absent, and the mutant chromosome can be recovered from the non-small-eyed siblings, who will be carriers.

We screened 233 pedigrees, each deriving from a single offspring of a mutagenized male. One pedigree produced only one deletion carrier out of 35 offspring in the test generation, and thus probably harbours a lethal mutation in the deletion region. The survival of this animal might be due to incomplete penetrance of the mutation or because meiotic recombination removed the mutation from the non-deletion chromosome. Using microsatellite markers flanking the deletion, we were able to follow the mutant chromosome in heterozygotes in subsequent generations. The intercrossing of heterozygotes resulted in the birth of 143 offspring, of which only nine were homozygotes (compared to an expected number of 36; $\chi^2=20.02$, $P<0.001$). Six of these did not survive to weaning; the remaining three were small and had an additional digit on one or both hindlimbs (see below). We expected that this line, MUTS1/14, would contain a mutation within the Pax6Sey-1H deletion.

Identification of a splice mutation in Dph4
The Pax6Sey-1H deletion is less than 2.5 Mb in length and encompasses all or part of 14 genes, from Ccde73 to Kcna4, which are candidates for the lethal mutation (R.E. and S.H.C., unpublished results) (Fig. 1B). While breeding heterozygous carrier mice, we found a recombination within this interval between a mutant and a wild-type chromosome, which established a smaller interval of about 1.4 Mb within which the mutation was located. This contained the ten genes from Ccde73 to Ddcd5. We sequenced the coding exons and splice sites of genes in this interval and found a T to A transversion at the splice donor site of intron 4 of a gene containing a J-domain and a CSL-zinc-finger domain (Fig. 2A). Analysis of the sequence databases revealed that this gene is the single mammalian orthologue of the yeast gene Dph4, which is required for diphthamide modification of eEF2 (Liu et al., 2004). We therefore called this mouse gene Dph4.

To investigate the consequences of the splice-site mutation, we performed reverse transcriptase (RT)-PCR on cDNA that was isolated from wild-type, heterozygous or homozygous embryos. Using primers within exons 3 and 5, we could amplify a 246-bp cDNA fragment encompassing exon 4 from wild-type mRNA. Upon amplifying the cDNA from homozygous mutant embryos, however, the PCR product was smaller by 69 bp, the size of exon 4, and DNA sequencing showed that this exon was absent from the cDNA. Both wild-type and mutant PCR products were amplified from heterozygous cDNA. Thus, the ethylisotiosurea (ENU)-induced mutation results in the skipping of exon 4 in the mutant mRNA (Fig. 2B,C).

We expressed the wild-type mouse DPH4 peptide as a fusion protein in Escherichia coli and raised an antibody in rabbits against the DPH4 component. Western blotting of proteins from wild-type embryos probed with this antibody revealed a band of approximately 17 kD, close to the predicted molecular mass of the protein (Fig. 2D). The exon-skipping resulting from the splice-site mutation should cause an in-frame deletion, which would produce a protein that was internally truncated by 23 amino acids in mutant and heterozygous embryos. However, western blot analysis of these
embryos did not detect a truncated protein (Fig. 2D). In particular, in homozygous embryos there was no protein of the predicted size (nor any other novel band). We conclude that the translation product of the internally deleted Dph4 mRNA must be unstable within the cell and is degraded. The mutant embryos therefore lack not only full-length DPH4 protein, but also lack the shortened mutant version and presumably lack all DPH4 function.

Loss of Dph4 results in lack of diphthamide modification of eEF2 and resistance to diphtheria toxin

We anticipated that the homozygous mutant embryos would lack the diphthamide modification of the eEF2 protein. Diphthamide modification of His715 results in an additional positive charge on eEF2, which can be detected by non-denaturing gel electrophoresis. Protein extracts from wild-type, mutant and heterozygous embryos were separated by native PAGE and probed on western blots with an antibody against eEF2. Fig. 2E shows that eEF2 from mutant embryos has a faster electrophoretic mobility than that from wild type, consistent with a charge of −1 relative to the protein from wild-type embryos; this is consistent with lack of diphthamide modification. Notably, the eEF2 from heterozygotes had a mobility that was identical to wild-type mice, which is consistent with the normal presence of diphthamide in these mutants.

We predicted that eEF2 from mutant cells would be resistant to ADP-ribosylation by DT and that the cells would be resistant to its toxic effects. Mouse cells are normally resistant to DT because of a lack of the surface receptor via which the B-subunit of the toxin gains access to the cell (Naglich et al., 1992; Stenmark et al., 1988). However, if the A-subunit of the toxin (DTA) is transfected into mouse cells, it triggers rapid cell death. We isolated embryonic fibroblasts (MEFs) from wild-type, mutant and heterozygous embryos. The MEFs were then transfected with the firefly luciferase gene, with luciferase plus the DTA gene, or with luciferase plus a vector-only plasmid. Co-transfection with DTA into wild-type or heterozygous cells resulted in cell death and, thus, a reduction of luciferase activity (Fig. 3). By contrast, co-transfection of DTA had no effect on luciferase activity in mutant cells, indicating that they lack the diphthamide target of DT (Fig. 3).

DPH4 protein localizes to the cytoskeleton

The DPH1 and DPH2 proteins interact in mammalian cells (Liu et al., 2004) and there is evidence from yeast that DPH3 also interacts with both of them (Fichtner, 2003), suggesting that there might be a multimeric protein complex that catalyzes diphthamide biosynthesis. The mouse DPH1 protein (also known as OVCA1) has been localized to the perinuclear region by transfection of an epitope-tagged gene (Chen and Behringer, 2001). Because DPH4 participates in the same biochemical pathway, we investigated whether it colocalized with DPH1. We co-transfected NIH-3T3 cells with a Myc-tagged Dph1 construct and FLAG-tagged Dph4 and localized both proteins by immunofluorescence. Surprisingly, whereas DPH1 was seen in a punctate, perinuclear pattern, in agreement with the published localization, DPH4 appears to localize to the cytoskeleton (Fig. 4A-C). To confirm this localization we stained cells that were transfected with FLAG-tagged Dph4 with
the cytoskeletal marker phalloidin, which indicated that the tagged DPH4 does indeed localize to the cytoskeleton (Fig. 4D-I).

Discussion

Five yeast genes have been identified, following saturation mutagenesis, as being required for diphthamide biosynthesis (Liu et al., 2004), and all five have sequence orthologues in mouse and humans. Mutations in four of these have been selected in CHO cells (Liu and Leppa, 2003; Liu et al., 2004; Moehring and Moehring, 1979; Moehring et al., 1980; Moehring et al., 1984; Nobukuni et al., 2005) and two have been subject to targeted mutagenesis in mouse (Chen and Behringer, 2004; Liu et al., 2006). Mutations in Dph4 have not been reported, to date, in higher eukaryotes and direct functional evidence for its role has been absent. We have presented a mouse mutation in Dph4 and functional evidence that the gene product is required for diphthamide biosynthesis.

eEF2 protein is essential for polypeptide elongation in protein synthesis, forming a complex with GTP and the ribosomes and catalyzing the GTP-dependent translocation of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site on the ribosome (Spahn et al., 2004). The function of diphthamide within eEF2 is not well understood. However, recent work has shown a requirement for diphthamide in the maintenance of translational fidelity. Structural studies have suggested a possible interaction between diphthamide and two universally conserved adenine residues on the ribosomal RNA that are essential for tRNA recognition at the A-site in the small ribosomal subunit. These two adenosines undergo a conformational change, switching from a stacked (closed) position in the absence of tRNA to a flipped-out (open) position in the presence of cognate tRNA. Jørgensen et al. (Jørgensen et al., 2005) proposed that diphthamide might be required for the stabilization of the stacked position of the adenosines, and therefore be essential for maintaining the correct reading frame during translocation across the ribosome. Studies using yeast mutant strains that lack either Dph2 or Dph5 show an increase in –1 frameshifting (Ortiz et al., 2006).

Biochemical studies indicate that DPH1-DPH4 catalyze the first step in diphthamide biosynthesis, the transfer of 3-amino-3-carboxypropyl from S-adenosylmethionine to the histidine. DPH5 catalyzes the subsequent trimethylation of the intermediate. DPH1 and DPH2 are related, but not mutually redundant, proteins that physically interact in both yeast and mammalian cells (Liu et al., 2004). Large-scale interaction studies in yeast have found mutual interactions between DPH1, DPH2 and DPH3, and between all of these three and eEF2. In addition, DPH5 also interacts with eEF2 (Collins et al., 2007; Gavin et al., 2006; Krogan et al., 2006). However, DPH4 has not been found to interact with any of these components. Our finding that at least some DPH4 localizes to the cytoskeleton, unlike DPH1, supports the model that a complex of DPH1-DPH3 directly interacts with eEF2 to promote the first stage of diphthamide synthesis. DPH5 might also separately bind eEF2 during the second stage. The role of DPH4 is not clear. DPH4 has a bipartite structure consisting of a J-domain and a so-called CSL

Table 1. Genotypes of embryos from an intercross of carriers of the Dph4 mutation on a C3H background

<table>
<thead>
<tr>
<th>Embryonic age</th>
<th>Wild type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
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<tr>
<td>E8.5</td>
<td>3</td>
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<td>E9.5</td>
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<td>E13.5</td>
<td>10</td>
<td>26</td>
<td>5</td>
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<td>E14.5 and older</td>
<td>16</td>
<td>36</td>
<td>9</td>
</tr>
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Fig. 5. The developmental consequences of Dph4 mutation. (A-D) Dph4 homozygous mutants are small in size. (A) E10.5 embryos: left, wild-type embryo; centre and right, two homozygous mutant embryos. (B) E11.5 embryos: left, wild type; right, homozygous mutant. (C) E12.5 and E13.5 embryos: left, E12.5 wild-type embryo; centre, E13.5 wild-type embryo; right, E13.5 homozygous mutant embryo, to illustrate the ~1-day development delay. (D) E13.5 embryos: left, wild type; centre and right, two homozygous mutant embryos. (E-G) Dph4 homozygous mutants have a preaxial polydactyly. (E) Hindfoot of an E14.5 homozygous embryo. (F) Hindfoot of an E16.5 homozygous embryo. (G) Stain.

The DPH1 gene was originally identified as a gene commonly deleted in human ovarian cancer, and mice heterozygous for a mutation of Dph1 have an increased incidence of various tumours (Chen and Behringer, 2004). We have not observed tumours in mice heterozygous for the Dph4 mutation, nor have tumours been reported in Dph3 heterozygous mice. It is possible that Dph1 has additional functions that act as tumour suppressors.

Materials and Methods

Mouse mutagenesis and husbandry

Animal studies were carried out under the guidance issued by the Medical Research Council in ‘Responsibility in the Use of Animals in Medical Research’ (July 1993) and licensed by the Home Office under the Animals (Scientific Procedures) Act 1986. Male BALB/c mice were dosed with ENU as described (Nolan et al., 2000) to mutagenize the spermatogonial stem cells. Following recovery of fertility, they were mated to Pax6<sup>flx<sup>Wnt</sup></sup> females and subsequently bred as in Fig. 1A. The mutant BALB/c chromosomal segment was followed through matings using the microsatellite markers D2Mit42 and D2Mit58, which flank the region deleted in Pax6<sup>flx<sup>Wnt</sup></sup>.

DNA sequencing

Candidate exons were amplified using primers designed to permit sequencing of the whole exon plus the consensice splice sites. BALB/c DNA was also sequenced to allow identification of SNPs compared with the reference C57BL6/J sequence.

RT-PCR

RNA was isolated from mouse embryos using the RNAagents Total RNA Isolation System (Promega). RT-PCR was carried out using the Access RT-PCR System (Promega) using the following primers from exons 3 and 5 of Dph4: 5′-CAAAGTGCAGATGTGCCA-3′ and 5′-GGAGACAGTGTATTTCCCACC-3′. Annealing was at 60°C.

Production of antiserum

A DPH4-GST fusion protein was made by cloning Dph4 cDNA into pGEX-KG and expression was induced by IPTG treatment in BL21 cells (Guan and Dixon, 1991). Following extraction by sonication, the fusion protein was bound to glutathione beads (GS4B Sepharose; Amersham Biosciences), washed and DPH4 was liberated by cleavage with thrombin. The purified protein was injected into rabbits by Diagnostics Scotland to generate antiserum.

Protein analysis of embryos

Individual E10.5 embryos were disrupted by pipetting in modified RIPA buffer followed by centrifugation to remove debris. The isolated proteins were either run on standard SDS-PAGE or on native (non-denaturing) PAGE, using precast 4-12% tris-glycine gels (Novex). Proteins were transferred to Hybond-P by electrophoretic blotting. The western blots were probed with the whole antiserum raised against DPH4 or a goat antibody against eEF2 (Santa Cruz, sc13004), both at a dilution of 1 in 500. Antibody reaction was detected using horse-radish-peroxidase-conjugated secondary antibodies: NA934VS goat anti-rabbit (Amersham) at 1 in 10,000 or sc2020, donkey anti-goat, (Santa Cruz) at 1 in 5000. Visualization used ECL-Plus (Amersham).

Given the ubiquity of the diphthamide modification, it is perhaps surprising that ablation of its synthesis is not cell lethal. More remarkable still is that mouse embryos that lack diphthamide synthesis, although retarded in growth, nevertheless undergo remarkable translation elongation factors, appears to be localized to the actin cytoskeleton (Bektas et al., 1994; Bektas et al., 1998; Shestakova, 1991), and this colocalization might permit at least transient interaction with DPH4.

The phenotype of our Dph4 mutants is similar to the Dph1-knockout mice, with developmental delay of about 1 day and prenatal lethality. We have shown a strong effect of genetic background such that, when outbred, occasional mice can survive beyond birth to maturity and fertility. It is very striking that both Dph1 and Dph4 mutants have a very similar preaxial polydactyly phenotype. It remains to be determined whether this is due to a defect in translation of a specific developmental factor or factors, or whether the overall growth retardation of the embryos results in a mismatch in timing of developmental events, which leads to the limb phenotype.

The J-domain is the crucial feature of the Dnaj, or Hsp40, family of proteins, which act as Hsp70 co-chaperones (Walsh et al., 2004). The J-domain interacts with Hsp70 and regulates its activity, inducing ATPase activity and altering substrate binding. Previous work on mouse DPH4, as mmDjC7, indicates that the J-domain is indeed functional in that it is able to stimulate the ATPase activity of the Hsp70 proteins Hsc70 and BiP (Kroczynska and Blond, 2001). Thus, DPH4 is likely to be a molecular chaperone and enable protein folding. Whether the substrate is one or more of DPH1, DPH2, DPH3 or eEF2, or indeed is another still to be identified substrate, remains to be established. It is notable that, in mammalian cells, some fraction of eEF2, along with other translation elongation factors, appears to be localized to the actin cytoskeleton (Bektas et al., 1994; Bektas et al., 1998; Shestakova, 1991), and this colocalization might permit at least transient interaction with DPH4.

The CSL-zinc-finger motif is found in only one other protein, DPH3, in which it comprises almost the entire molecule, zinc finger. The CSL-zinc-finger motif is found in only one other protein, DPH3, in which it comprises almost the entire molecule, zinc finger. The CSL-zinc-finger motif is found in only one other protein, DPH3, in which it comprises almost the entire molecule, zinc finger. The CSL-zinc-finger motif is found in only one other protein, DPH3, in which it comprises almost the entire molecule, zinc finger. The CSL-zinc-finger motif is found in only one other protein, DPH3, in which it comprises almost the entire molecule, zinc finger.
Mouse embryonic fibroblasts
MEFs were isolated by dissecting individual E13.5 embryos to remove the head and internal organs, incubating in dispase and dispersing cells using a syringe needle. DNA was isolated from the extracellular membranes of each embryo and sequenced to determine the Dhph4 genotype. The cells were cultured in DMEM with 10% fetal calf serum.

Diphtheria-toxin-sensitivity assay
DNA was introduced into MEFs by electroporation using the Digital Bio Microporator, with parameters recommended by the manufacturers. The pGL3-P luciferase reporter plasmid (Promega) was introduced into about 4×10^6 cells per electroporation. To assay the sensitivity to DT, the DTA subunit gene, driven by the PGK promoter, was co-electroporated with the pGL3-P plasmid at a molar ratio of approximately 3:1. Control co-electroporations used pBluescript vector at the same molar ratio. Following overnight incubation, luciferase activity was measured using the Luciferase Assay System (Promega) and a Lumat LB9507 luminometer (Berthold). Electroporations and luciferase assays were performed in triplicate.

Immunofluorescence
Subcellular localization of tagged proteins was carried out in NIH-3T3 cells, following transfection with Lipofectamine 2000 (Invitrogen). FLAG-tagged Dhph4 was generated by cloning the cDNA into p3×FLAG-CMV10 (Sigma). Mouse DHPH1 tagged with myc was a gift of Chun-Ming Chen (Chen and Behringer, 2001). Following transfection, the tagged proteins were detected by anti-FLAG mouse or Alexa-Fluor-conjugated anti-myc, sc789 (Santa Cruz) at 1 in 100. Following transfection, the tagged proteins were detected by anti-FLAG mouse monoclonal F-1365 (Sigma) at 1 in 200, followed by anti-mouse Texas Red (Stratech) or Alexa-Fluor-conjugated anti-myc, sc789 (Santa Cruz) at 1 in 100.

Embryos
Embryos were dissected into chilled PBS before fixation overnight in 4% PFA. Extraembryonic membranes or tails were used as the source of DNA for genotyping. Embryos were dissected into chilled PBS before fixation overnight in 4% PFA. DNA was introduced into MEFs by electroporation using the Digital Bio Microporator, with parameters recommended by the manufacturers. The pGL3-P luciferase reporter plasmid (Promega) and a Lumat LB9507 luminometer (Berthold). Electroporations and luciferase assays were performed in triplicate.

References


