Identification of the Proteins Required for Biosynthesis of Diphthamide, the Target of Bacterial ADP-Ribosylating Toxins on Translation Elongation Factor 2†

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Diphthamide, a posttranslational modification of translation elongation factor 2 that is conserved in all eukaryotes and archaebacteria and is the target of diphtheria toxin, is formed in yeast by the actions of five proteins, Dph1 to -5, and a still unidentified amidating enzyme. Dph2 and Dph5 were previously identified. Here, we report the identification of the remaining three yeast proteins (Dph1, -3, and -4) and show that all five Dph proteins have either functional (Dph1, -2, -3, and -5) or sequence (Dph4) homologs in mammals. We propose a unified nomenclature for these proteins (e.g., HsDph1 to -5 for the human proteins) and their genes based on the yeast nomenclature. We show that Dph1 and Dph2 are homologous in sequence but functionally independent. The human tumor suppressor gene *OVCA1*, previously identified as homologous to yeast *DPH2*, is shown to actually be *HsDPH1*. We show that *HsDPH3* is the previously described human diphtheria toxin and *Pseudomonas* exotoxin A sensitivity required gene 1 and that *DPH4* encodes a CSL zinc finger-containing DnaJ-like protein. Other features of these genes are also discussed. The physiological function of diphthamide and the basis of its ubiquity remain a mystery, but evidence is presented that Dph1 to -3 function in vivo as a protein complex in multiple cellular processes.

Diphthamide is a unique posttranslationally modified histidine residue found only in translation elongation factor 2 (eEF-2). It is conserved from archaebacteria to humans and serves as the target for diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) (9). DT and ETA specifically catalyze the transfer of ADP-ribose from NAD^+ to diphthamide on eEF-2 (Fig. 1A), thus inactivating eEF-2, halting cellular protein synthesis, and causing cell death. Although diphthamide has been found in all eukaryotic organisms and archaebacteria, it is not present in eubacteria. Thus, DT and ETA have evolved a specific mechanism for targeting eukaryotic protein-synthetic machinery without inactivating the analogous elongation factor (EF-G) present in the bacterial pathogens that produce them. Despite the fact that this modification was first identified nearly 25 years ago (32), no obvious role for diphthamide has yet been identified in the normal physiology of the eukaryotic cell. In fact a number of mutants have been identified in both yeast (*Saccharomyces cerevisiae*) and CHO cells that fail to make diphthamide, yet these mutants do not exhibit obvious phenotypes (7, 26).

The biosynthesis of diphthamide represents one of the most complex posttranslational modifications. The biosynthesis is accomplished by stepwise additions to the His^{715} (His^{699} in yeast) residue of eEF-2 (6, 25, 26), beginning with transfer of the 3-amino-3-carboxypropyl group of *S*-adenosylmethionine (AdoMet) to the imidazole C-2 of the precursor histidine residue (Fig. 1A). Trimethylation of the resulting amino group follows, using AdoMet as the methyl donor, to produce diphthine. The final step is an ATP-dependent amidation of the carboxyl group, yielding diphthamide. This complex pathway is amenable to genetic analysis, and mutants defective in diphthamide biosynthesis have been isolated in both CHO cells and yeast cells by selection for resistance to the actions of DT and ETA. The previously defined mutations are recessive and have been shown to sort into five complementation groups (Dph1, Dph2, Dph3, Dph4, and Dph5) in yeast (7) and three complementation groups (CG-1, CG-2, and CG-3) in CHO cells (26). Biochemical analyses suggested that the gene products lacking in the yeast *dph1*, *dph2*, *dph3*, and *dph4* mutants and the CHO complementation groups CG-2 and CG-3 are involved directly or indirectly in the first step of diphthamide biosynthesis—the transfer of 3-amino-3-carboxypropyl from AdoMet to the imidazole C-2 of the precursor histidine residue in eEF-2— whereas the genes lacking in *dph5* and CG-1 act in the trimethylation of the diphthamide intermediate (Fig. 1A) (6, 7, 22, 26). No mutations in the final amidation step were obtained in the previous selections because diphthine can be ADP ribosylated, although slowly, and therefore amidation-deficient mutants would not be resistant to DT (26).

Mattheakis and colleagues identified the *S. cerevisiae DPH2* and *DPH5* genes by using a genetic-complementation ap-

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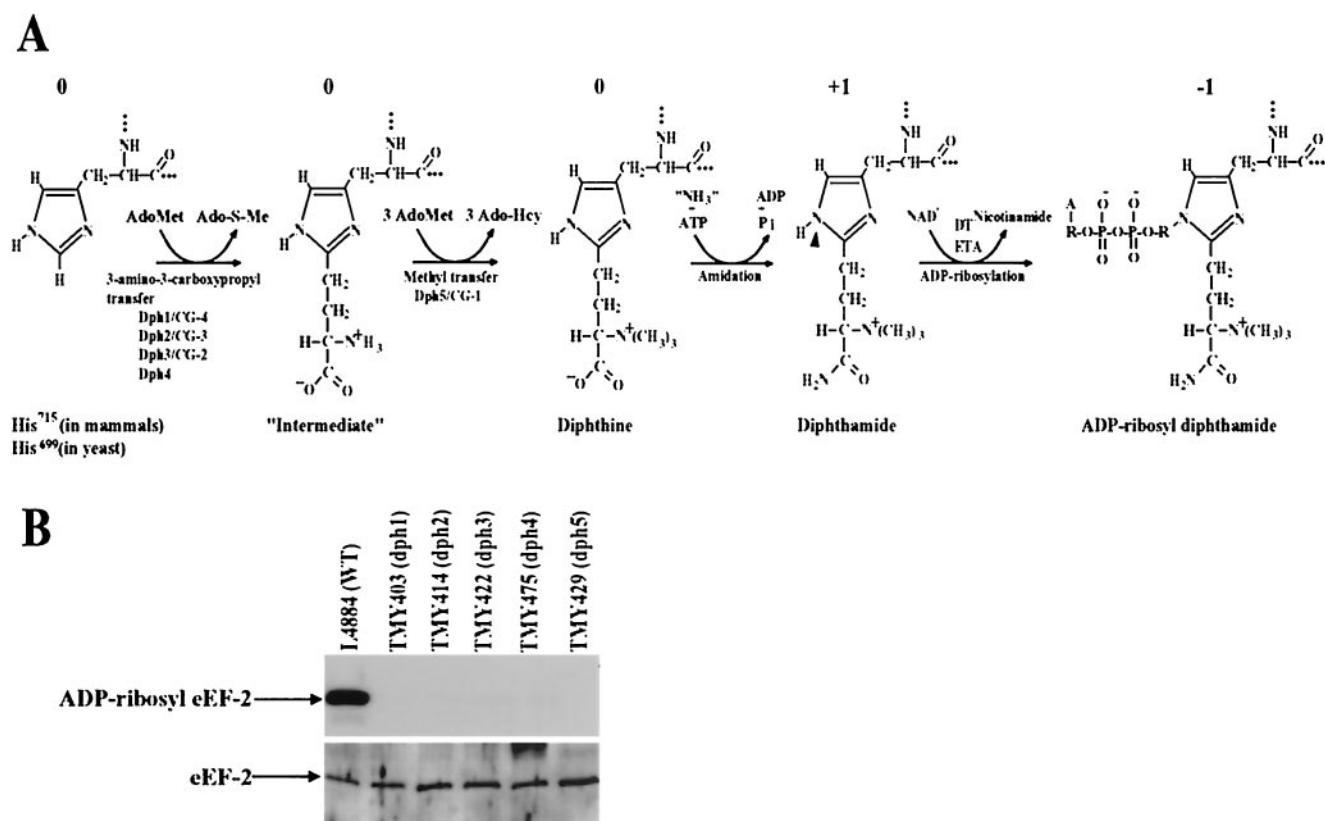§ Present address: Microbia, Inc., Cambridge, MA 02141.

FIG. 1. *DPH1* to *DPH5* genes are required for diphthamide biosynthesis. (A) Diphthamide biosynthesis and ADP ribosylation. N-1 (arrowhead) of the histidine imidazole ring of diphthamide is the site for ADP ribosylation by DT and ETA. Ado-S-Me, methylthioadenosine; Ado-Hcy, *S*-adenosylhomocysteine. In ADP-ribosyl diphthamide, A, adenine moiety; R, ribosyl moiety. The relative net charges of the modified His side chains are indicated above each species. (B) Precise deletions of each of *DPH1* to *DPH5* genes result in the yeast mutants being completely resistant to ADP ribosylation by DT. (Top) Yeast cell lysates were incubated with [adenylate-^{32}P]NAD and DT at room temperature for 30 min. The reactions were terminated by boiling the lysates in SDS sample buffer, and the samples were analyzed by SDS-PAGE, followed by autoradiography. (Bottom) Deletions of each of the *DPH1* to *DPH5* genes do not affect overall expression of eEF-2 protein as evidenced by Western blot analysis using an anti-eEF-2 antibody (a gift of Angus Nairn, Rockefeller University).

proach. *DPH2* encodes a 534-residue protein with no con-served domains that might suggest a function (23). *DPH5* en-codes a 300-residue AdoMet-dependent methyltransferase (22). Recently, the gene impaired in a CG-2 CHO mutant was shown to encode an 82-residue peptide, and the gene was named *DESR1*, for DT and ETA sensitivity required gene 1 (19). Its functional homolog in yeast, the *Kluyveromyces lactis* toxin-insensitive gene 11 (*KTI11*), was originally identified as a gene regulating the sensitivity of *S. cerevisiae* to zymocin (11), implicating *DESR1/KTI11* in multiple biological processes.

In the work presented below, we report the identification of the remaining three genes, *DPH1*, *DPH3*, and *DPH4*, from yeast and the three genes impaired in the CG-1, CG-3, and CG-4 diphthamide-deficient CHO mutants that are required for diphthamide biosynthesis. We show that all five Dph pro-teins are highly conserved among eukaryotic species, under-scoring the apparent evolutionary importance of the unique diphthamide modification to eEF-2. We propose a unified no-menclature for these proteins (i.e., HsDph1 to -5 for the hu-man proteins) and their genes based on the yeast nomencla-ture. We show that *DPH1*, the gene also identified as impaired in a CG-4 CHO mutant, appears to be identical to the recently

identified *OVCA1* (for ovarian cancer gene 1), a tumor sup-pressor gene (5) that plays a crucial role in the regulation of cell proliferation, embryonic development, and tumorigenesis. Dph1 and Dph2 are homologous in sequence and associate in vivo but are functionally independent. Other features of the diphthamide biosynthesis genes are also discussed.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The yeast strains used and con-structed in this study are listed in Table 1. Yeast mutants representing each of the five Dph complementation groups (Dph1, Dph2, Dph3, Dph4, and Dph5) de-fined previously (7) were kindly provided by James W. Bodley (University of Minnesota, Minneapolis). The *URA3*-marked plasmid pLMY101 encoding the DT F2 fragment (under the transcriptional control of the yeast *GAL1* promoter) (22) was a kind gift of R. John Collier (Harvard Medical School, Boston, Mass.). Precise deletions of each of the *DPH* genes were constructed in a W303 genetic background by PCR amplification of a selectable marker flanked with the rele-vant *DPH* sequences and transformation into strain L4884 to yield TMY403, TMY414, TMY422, TMY475, and TMY429 (Table 1). Strain 10556-6B is iso-genic with this series and was used as a wild-type strain to control for the tryptophan prototrophy associated with the *dph1*, *dph4*, and *dph5* knockouts.

Yeast strains were grown using standard methods (13) in rich medium (yeast-peptone-dextrose or yeast-peptone-dextrose supplemented with 0.3 mM ade-nine) or synthetic complete (SC) medium with one or more nutrients omitted as

TABLE 1. Yeast strains used in this study

Strain	Genotype[a]	Source or reference
Dph1/TMY288	*MAT**a** dph1-1 ura3-52 leu2-3,112 his3Δ1 trp1-289 met2 arg4*	7
Dph2/TMY289	*MAT**a** dph2-1 ura3-52 leu2-3,112 his3Δ1 trp1-289 met2 arg4*	7
Dph3/TMY290	*MAT**a** dph3-1 ura3-52 leu2-3,112 his3Δ1 trp1-289 met2 arg4*	7
Dph4/TMY291	*MAT**a** dph4-1 ura3-52 leu2-3,112 his3Δ1 trp1-289 met2 arg4*	7
Dph5/TMY292	*MAT**a** dph5-1 ura3-52 leu2-3,112 his3Δ1 trp1-289 met2 arg4*	7
L5146	*MAT**a** ura3-52 leu2-3,112 his3 trp1Δ63 ade2 lys2Δ201*	Fink collection
TMY196	L5146 carrying pLMY101, a *URA3*-marked plasmid encoding DT F2 fragment, which is under the transcriptional control of the yeast *GAL*1 promoter (22)	This study
10560-4A	*MAT**a** ura3-52 leu2::hisG his3::hisG trp1::hisG*	Fink collection
TMY203	10560-4A carrying pLMY101	This study
10560-6B	*MATα ura3-52 leu2::hisG his3::hisG trp1::hisG*	Fink collection
TMY204	10560-6B carrying pLMY101	This study
10560-23C	*MATα ura3-52 leu2::hisG his3::hisG*	Fink collection
TMC58-6D	*MAT**a** dph3-1 ura3-52 leu2 his3 trp1-289,* resulting from a cross between 10560-23c and Dph3/TMY290	This study
TMY345	TMC58-6D carrying pLMY101	This study
L4884	*MATα ura3-1 leu2-3,112 his3-11,15 ade-2 trp1Δ63 can1-100*	Fink collection
TMY403	Same as L4884, but *dph1Δ::TRP1*	This study
TMY414	Same as L4884, but *dph2Δ::HIS3*	This study
TMY422	Same as L4884, but *dph3Δ::HIS3*	This study
TMY475	Same as L4884, but *dph4Δ::TRP1*	This study
TMY429	Same as L4884, but *dph5Δ::TRP1*	This study
10556-6B	*MATα ura3-1 leu2-3,112 his3-11,15 ade-2 can1-100*	Fink collection

[a] *MAT**a**,* a mating type; *MATα,* alpha mating type.

indicated (e.g., SC minus Ura) and either glucose or galactose as a carbon source. Solid medium was prepared by adding agar to 2%. Unless otherwise indicated, all incubations were done at 30°C on plates or in shaking flasks.

ADP ribosylation assay. The assay for ADP ribosylation of eEF-2 in yeast or CHO cell extracts was described previously (19, 22).

CHO cell lines, cell culture, and transfection. CHO K1 parental cells and their DT-resistant mutants RPE3b (CG-1), RPE33d (CG-3), and RPE22e (CG-3) (25, 26) were kind gifts from Gary Ward (University of Vermont). CHO cell mutant PR303 (CG-4) was isolated by retroviral insertional mutagenesis from its parental CHO WTP4 cells (19). All CHO cells were grown in α minimal essential medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 50 μg of gentamicin/ml, and 25 mM HEPES.

The mouse *DPH1, DPH2,* and *DPH5* cDNA fragments were isolated by reverse transcription-PCR from mouse liver total RNA. The mouse *DPH1* cDNA fragment was amplified using the 5′ primer Dph1-5 (AAG**TGTACA**TGGCGG CGCTGGTTGTGTCCGAGACT; the BsrGI site is in boldface, and the start codon is underlined) and a 3′ primer, Dph1-3 (TCA**TGATCA**GGGAGCCGGC GAAGTAGCCTTCT; the antisense of the stop codon is underlined; the BclI site is in boldface). The mouse *DPH2* cDNA fragment was amplified using the 5′ primer Dph2-5 (AAG**TGTACA**TGGAGTCTACGTTCAGCAGCCCTGC; the BsrGI site is in boldface, and the start codon is underlined) and a 3′ primer, Dph2-3 (TCA**TGATCA**GCTGCTCCCCTCATCCTCGTAGGC; the antisense of the stop codon is underlined, and the BclI site is in boldface). The mouse *DPH5* cDNA fragment was amplified using the 5′ primer Dph5-5 (AAG**CGTA CG**ATGCTTTACTTGATCGGCGTTGGGCCTGGGA; the BsiWI site is in boldface, and the start codon is underlined) and a 3′ primer, Dph5-3 (TCA**TG ATCA**GAGTCCATCAGTACTCTGGGATTC; the antisense of the stop codon is underlined, and the BclI site is in boldface). The amplified cDNA fragments were then digested by BsrGI and BclI in the cases of mouse *DPH1* and mouse *DPH2* and by BsiWI and BclI for mouse *DPH5* and cloned between the BsrGI and BamHI sites of pIRESHgy2B (catalog no. 6939-1; Clontech). This bicistronic mammalian expression vector contains an attenuated version of the internal ribosome entry site of the encephalomyocarditis virus, which allows both the gene of interest and the hygromycin B selection marker to be translated from a single mRNA. The expression plasmids were confirmed by DNA sequencing and were transfected into CHO cells by using Lipofectamine 2000 reagent (Life Technologies), and the stably transfected cells were selected by growth in hygromycin B (500 μg/ml) for 2 weeks. The expression plasmids used in this study are listed in Table S1 in the supplemental material.

Cytotoxicity assays. DT was produced as described previously (4). The CHO cell cytotoxicity assay with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was performed as described previously (19).

Western blot analysis. CHO cells grown in 24-well plates were lysed by adding 100 μl of modified RIPA lysis buffer containing protease inhibitors/well (19). Portions (20 μl) of the cell lysates were incubated with or without 300 ng of fully nicked DT at room temperature for 30 min and then were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native PAGE using 4 to 12% Tris-glycine gradient gels (Novex, San Diego, Calif.). Prior to being loaded, the cell lysates were boiled for 5 min in 1× SDS sample buffer (Novex) for SDS-PAGE or were incubated for 10 min at room temperature in 1× native sample buffer (Novex) for native PAGE. The proteins were then transferred to nitrocellulose membranes overnight, followed by Western blotting using a goat antibody (catalog no. sc-13004; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) directed to a linear peptide at the carboxyl terminus of eEF-2.

Coimmunoprecipitation assay. The amino-terminal hemagglutinin (HA) epitope (MYPYDVPDYA)- or Myc epitope (MASMQKLISEEDL)-tagged mouse *DPH1,* mouse *DPH2,* and mouse *DPH5* cDNA fragments were amplified by using the pairs of 5′-tag-coding primers (with the sequence coding for the tags and the amino termini of the Dph proteins) and 3′ primers listed above. These tagged cDNA fragments were then cloned into the expression plasmid pIRESHgy2B, resulting in the HA- or Myc-tagged Dph protein expression plasmids (see Table S1 in the supplemental material), which were confirmed by sequencing. Combinations of these plasmid constructs (3 μg each), in some cases containing HsDph3-HA (19), were transfected using Lipofectamine 2000 reagent into CHO K1 cells grown in T25 flasks. Cell lysates were prepared using modified RIPA lysis buffer containing protease inhibitors after 24 h and immunoprecipitated using an agarose-conjugated anti-Myc antibody (catalog no. sc-789AC; Santa Cruz Biotechnology, Inc.). The precipitates were analyzed by Western blotting using an anti-HA antibody (catalog no. sc-7392; Santa Cruz Biotechnology, Inc.).

RESULTS

Isolation of diphthamide-deficient yeast mutants and cloning of the corresponding genes. Diphthamide-deficient mutants were selected, based on their resistance to the conditional intracellular expression of the DT F2 fragment, as described previously (22) from pools of cells that were either transposon mutagenized or unmutagenized (spontaneous mutants). Transposon mutants were generated by transformation of a transposon-mutagenized genomic library (3) into strain

TABLE 2. Frequency of transposon-induced and spontaneous DT-resistant yeast mutants falling in each complementation group

Complementation group	No. (%) of transposon mutants	No. (%) of spontaneous mutants	Total no. (%)	No. (%) of *dph* mutants isolated by Chen et al. (7)
Dph1	157 (32)	92 (25)	249 (29)	17 (55)
Dph2	1 (0.2)	52 (14)	53 (6)	9 (29)
Dph3	0 (0)	46 (13)	46 (5)	1 (3)
Dph4	134 (28)	136 (37)	270 (32)	3 (10)
Dph5	192 (40)	37 (10)	229 (27)	1 (3)
Total	484 (100)	363 (100)	847 (100)	31 (100)

TMY196 (Table 1), which carries a *URA3*-marked plasmid, pLMY101 (22), expressing the DT F2 fragment (22). The 75,000 independent transformants obtained were pooled, and a total of 1.1×10^6 CFU was plated on 10 SC plates minus Ura-Leu with galactose as the sole carbon source to induce DT expression. Colonies that arose were designated DTR (DT resistant) and patched to master plates for subsequent analysis. To determine whether these DTR survivors belonged to the five complementation groups previously identified (7), they were crossed with *dph1*, *dph2*, *dph3*, *dph4*, and *dph5* strains (Table 1). If the resultant diploid was able to grow (no complementation) on a galactose plate, then the parent strains were considered to be in the same complementation group. Failure to grow indicated a different complementation group. Through this analysis, 484 recessive mutants were identified, and each could be classified into one of the complementation groups Dph1, Dph2, Dph4, and Dph5 (Table 2), with none identified as *dph3* mutants.

Because *DPH2* and *DPH5* were identified previously, only *dph1* and *dph4* strains were further analyzed. Crossing representative transposon-induced clones of the Dph1 and Dph4 complementation groups with wild-type strains resulted in 2:2 segregation, consistent with mutations at single loci. In each case, the *LEU2* transposon marker cosegregated with the DTR phenotype. To identify the targeted genes, several transposon insertion sites were sequenced and found to correspond to insertions in open reading frame (ORF) *YIL103w* in *dph1* strains and ORF *YJR097w* in *dph4* strains. The *DPH1* and *DPH4* gene identities were further confirmed by precisely deleting the *YIL103w* and *YJR097w* ORFs using a PCR-mediated deletion approach (1), resulting in deletion strains TMY403 and TMY475 (Table 1). Lysates of these strains had no DT-catalyzed ADP-ribose acceptor activity (Fig. 1B). The properties of these *DPH* genes and their relationships to mammalian genes from these and later analyses are summarized in Table 3.

In parallel with the transposon mutagenesis approach, spontaneous DT-resistant mutants were selected by plating $\sim 10^8$ cells of the pLMY101-carrying strains, TMY203 and TMY204 (Table 1), on SC plates minus Ura supplemented with galactose to induce expression of the DT gene. The resulting DTR clones were analyzed as described above. A total of 363 recessive DT-resistant mutants were obtained, and all of them were found to belong to the five known complementation groups (Table 2). In contrast to the transposon-induced mutants, the

TABLE 3. Summary of yeast and mammalian genes involved in diphthamide biosynthesis

Dph protein	Role in diphthamide biosynthesis	Yeast			Mammalian				% Identity between:	
		Gene name (ORF)	Protein description and localization[a]	Representative mutant strains	Gene name (Unigene identifier)	Protein (mouse) description	CHO CG[b]	CHO mutants	Yeast and mouse	Mouse and human
Dph1	3-Amino-3-carboxypropyl transfer	*DPH1 (YIL103w)*	425 aa; 16% identity to ScDph2; cytoplasm	TMY288, TMY403	*DPH1, OVCA1, Dph2L1,* (Mm.298543)	438 aa; tumor suppressor; 19% identity to MmDph2	CG-4	PR303	49	86
Dph2	3-Amino-3-carboxypropyl transfer	*DPH2 (YKL191w)*	534 aa; 16% identity to ScDph1; cytoplasm	TMY289, TMY414	*DPH2, Dph2L2* (Hs.324830)	489 aa; 19% identity to MmDph1	CG-3	PRE33d, PRE22e	22	83
Dph3	3-Amino-3-carboxypropyl transfer	*DPH3, KTI11,* (YBL071-wA)	82 aa; CSL zinc finger domain; cytoplasm, nucleus	TMY290, TMY422	*DPH3, DESR1*	82 aa; CSL zinc finger domain	CG-2	PIR2D17, PR72[c], PR201[c]	36	96
Dph4	Unknown	*DPH4 (YJR097w)*	172 aa; DnaJ domain, CSL zinc finger domain; cytoplasm, nucleus	TMY291, TMY475	*DPH4, DjC7?*	MmDjC7 193aa or 148 aa isoform; DnaJ domain, CSL zinc finger domain			22	83
Dph5	Trimethylation	*DPH5 (YLR172c)*	300 aa; diphthine methyltransferase; cytoplasm	TMY292, TMY429	*DPH5* (Dm.2376)	281 aa; diphthine methyltransferase	CG-1	RPE3b	55	88

[a] See reference 14. aa, amino acids.
[b] CG, complementation group.
[c] See reference 19.

spontaneous mutants included a number of *dph3* mutants (Table 2). To clone the *DPH3* gene, TMY345, a *dph3* strain harboring pLMY101 (Table 1), was transformed with a yeast genomic library (30) on SC plates minus Ura, using glucose as the carbon source. The transformants were screened by replica plating on SC plates minus Ura, using galactose as the carbon source to induce expression of DT. The two DT-sensitive colonies that were isolated contained overlapping genomic fragments. Subcloning and complementation analysis revealed that the *dph3*-complementing activity was present on a 2.4-kb SacI-NarI restriction fragment containing the ORF *YBL071w-A*. Precise deletion of the genomic copy of this gene resulted in strain TMY422 (Table 1), which had no DT-catalyzed ADP-ribose acceptor activity (Fig. 1B).

Identification of the genes required for diphthamide biosynthesis in CHO cells. DT-resistant CHO mutants were isolated previously by several groups, including our own, so as to identify mechanisms by which ADP-ribosylating toxins damage eukaryotic cells. The first set of such mutants (24–26) were sorted by cell fusion methods into three complementation groups, CG-1 to CG-3. The CHO diphthamide-deficient mutants we isolated fell into these complementation groups (19), with the exception of a mutant designated CHO PR303, which defines an additional complementation group, CG-4, discussed below. We recently identified the gene mutated in a CG-2 mutant as *DESR1*, the homolog of yeast *KTI11* (*YBL071w-A*) (19). As noted above, *dph3* mutants have mutations in *YBL071w-A*, showing that *DPH3* corresponds to the gene mutated in CG-2 (*DESR1*, referred to as *DPH3* hereafter). We also identify below the genes impaired in the CG-1, CG-3, and CG-4 mutants and associate all the CHO and yeast complementation groups, with the results summarized in Table 3.

Yeast *DPH1* (*YIL103w*) encodes a 425-residue protein with no obvious structural motifs but with significant amino acid sequence similarity to yeast Dph2 (ScDph2; 16% amino acid identity) (Fig. 2A and B). Despite the sequence similarity, Dph1 and Dph2 are not interchangeable in yeast, as mutations in either *DPH1* or *DPH2* block diphthamide biosynthesis and there is no apparent cross-complementation. Higher-eukaryotic genomes also contain a pair of *DPH1*- and *DPH2*-related genes. Although there is similarity within the pairs of gene products in each species, sequence alignment and clustering reveals that there are two distinct sequence groups—one corresponding to Dph1 and one corresponding to Dph2 (Fig. 2A and B). Sequence similarity within putative Dph1 or Dph2 clusters (e.g., ScDph1 and MmDph1 [mouse Dph1] show 49% identity) is significantly higher than that between the pairs in the same organism (e.g., ScDph1 and ScDph2 show 16% identity) (Fig. 2A and B). Although these relationships are clear in hindsight, the sequence similarity between Dph1 and Dph2 has led to confusing nomenclature so that the human *DPH1* gene has been designated *DPH2L* or *DPH2L1* (28). Therefore, for clarity, we have elected to use a consistent naming convention based on the yeast sequences, with alternate identifiers detailed in Table 3. The species is indicated by a two-letter abbreviation: ScDph1, MmDph1, and HsDph1 refer to Dph1 proteins from *S. cerevisiae* (yeast), *Mus musculus* (mouse), and *Homo sapiens* (human), respectively.
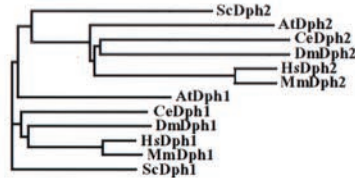
ScDph1 shows 49% identity with both MmDph1 and HsDph1 (human Dph1), whereas ScDph2 shows 22% identity with MmDph2 and HsDph2 (Fig. 2B). The *DPH1* genes of mouse and human origins were recently identified as tumor suppressors (*OVCA1*) (5). To explore the functions of mammalian Dph1 and Dph2 in diphthamide biosynthesis, the mouse cDNA sequences encoding these proteins were cloned into the bicistronic mammalian expression vector pIRESHyg2b. The resulting expression plasmids were then transfected into CG-4 CHO mutant PR303 cells and CG-3 CHO mutant PRE22e and PRE33d cells (Table 4). Stable tranfectants were directly evaluated for sensitivity to DT, and some were expanded as individual clones for further analysis. The majority of the colonies arising from PR303 cells transfected with *MmDPH1* and the majority of the colonies from RPE22e and RPE33d cells transfected with *MmDPH2* regained the parental sensitivity to DT (Table 4). In contrast, PR303 cells transfected with *MmDPH2*, and RPE22e and RPE33d cells transfected with *MmDPH1*, remained toxin resistant (Table 4). Furthermore, the representative individually expanded clones of PR303 cells transfected with *MmDPH1* and REP33d cells transfected with *MmDPH2* were fully sensitive to DT, just like their parental CHO WTP4 and CHO K1 cells (Fig. 3A and B). These results demonstrated that *DPH1* and *DPH2* are essential for diphthamide biosynthesis in mammals and also revealed that they are the genes impaired in the CG-4 and CG-3 CHO mutants, respectively. In agreement with the results from yeast, we showed that although MmDph1 and MmDph2 show 19% identity, they are functionally distinct and thus must perform different biochemical functions in diphthamide biosynthesis.

CHO RPE3b is a CG-1 diphthamide-deficient CHO mutant that was shown to be defective in AdoMet-dependent diphthine methyltransferase activity (26), the same activity lacking in yeast *dph5* strains (7, 22). Dph5 proteins are highly evolutionarily conserved, with the human and mouse sequences (GenBank accession numbers NP_057042 and BAB26968, respectively) showing 50 and 55% identity, respectively, with the yeast protein. To determine the role of mammalian Dph5 in diphthamide biosynthesis, the *MmDPH5* cDNA was isolated and expressed in CHO RPE3b using the same approach described above. The results showed that *MmDPH5* could reverse the toxin-resistant phenotype of RPE3b cells (Table 4 and Fig. 3B), demonstrating the conserved function of mammalian Dph5 in the trimethylation step of diphthamide biosynthesis and confirming that the gene impaired in CG-1 diphthamide-deficient CHO mutant is *DPH5*.

Identification of diphthamide biosynthetic intermediates in CHO mutants. DT inactivates eEF-2 by catalyzing the covalent attachment of the ADP-ribose moiety (containing two negatively charged phosphate groups) of NAD^+ to the N-1 position of the histidine imidazole ring of diphthamide (Fig. 1A). Therefore, the ADP-ribosylated eEF-2 has two added negative charges. The increased negative charge of the modified eEF-2 causes it to migrate faster than the non-ADP-ribosylated eEF-2 during gel electrophoresis under nondenaturing conditions (Fig. 3C). No differences were seen on a denaturing SDS gel (Fig. 3C). This analysis further confirmed that eEF-2 from parental CHO K1 and CHO WTP4 cells could be efficiently ADP ribosylated whereas that from the RPE3b (CG-1; Dph5), RPE33d (CG-3; Dph2), and PR303 (CG-4; Dph1) CHO mutant cells could not (Fig. 3C). The eEF-2 from Dph1- and

A

ScDph2
AtDph2
CeDph2
DmDph2
HsDph2
MmDph2
AtDph1
CeDph1
DmDph1
HsDph1
MmDph1
ScDph1

B

```
MmDph1  MAALVVSET--------------------AEPGS-RVGPGRGRISRGRLANQIPPEVL
ScDph1  MSGSTESKKQPRRRFIGRKSGNSNNDKLTTVAENGNEIIHKQKSRIALGRSVNHVPEDIL
ScDph2  --------------------------MEVAPALSTTQSDVAFQKVETHEIDRSSY
MmDph2  --------------MESTFSSPAEAALQREACGVPGQFTPPEDL

MmDph1  NNPQLQAAVQVLPSNYNFEIPKTLWRIQQAQAKK-VALQMPEGLLLFACTIVDILERFT-
ScDph1  NDKELNEAIKLLPSNYNFEIHKTVWNIRKYNAKR-IALQMPEGLLIYSLIISDILEQFC-
ScDph2  LGPCYNSDELMQLISAYYNVEPLVGYLEQHPEYQNVTLQFPDDLIKDSSLIVRLLQSKFP
MmDph2  DR--------VYELERVTKFVCDLGCQR-VTLQFPDQLLGDAGAVAARLEEVT-

MmDph1  --EAEVMVMGDVTYGACVDDFTARALGVDFLVHYGHSCLVPMDTSVQDFRVLYVFVDTR
ScDph1  --GVETLVMGDVSYGACCIDDFTARALDCDFIVHYAHSCLVPID--VTKIKVLYVFVTIN
ScDph2  HGKIKFWVLADTAYSACCVDEVAAEHVHAEVVVHFGDACLNAIQ----NLPVVYSFGTPF
MmDph2  --GAKMFILGDTAYGSCCVDVLGAEQAGAQALVHFGPACLSPPA---SQLPITFVLGQRP

MmDph1  IDTAHLLDSVRLTFT-PGSSLALVSTIQFVSTLQAAAQELKAD----YHTSVPQCKPLSP
ScDph1  IQEDHIIKTLQKNFP-KGSRIATFGTIQFNPAVHSVRDKLLNDEEHMLYIIPPQIKPLSR
ScDph2  LDLALVVENFQRAFPDLSSKICLMANAPFSKHLSQLYNILKGD-LHYTNIIYSQVNTSAV
MmDph2  VALELCAKAFEAQNPDPTAPVVLLSEPACAHALEPLAMLLLPKYQDLLISRPALPLPVGS

MmDph1  GEILGCTSPRLSKE--------VEAVVYLGDGRFHLES---------VMIANPNIPAY
ScDph1  GEVLGCTSERLDKEQ--------YDAMVFIGDGRFHLES---------AMIHNPEIPAF
ScDph2  EEKFVTILDTFHVPEDVDQVGVFEKNSVLFQFPDDKADNISPEDYHLFHLTTPQDPRLLYL
MmDph2  PSSQPESLERFGRCFPLNPGRRLEEYGAFYVCASQASSDSSLDPDLSRLLLGWTPGRFFF

MmDph1  RYDPYGKVLSREYYD-----HQRMQATRQEATAAARSAKSWGLILGTLGRQGSPKILEHL
ScDph1  KYDPYNRKFTREGYD-----QKQLVEVRAEAIEVARKGKVFGLILGALGRQGNLNTVKNL
ScDph2  STVFQSVHIFDPALYGPVTGPFPSLMRHKYKMHVARTAGCIGILVNTLSLRNTRETINEL
MmDph2  SCCFDTGQTQDQGAK-----AGRLRARRLYLIERARDARVVGLLAGTLGVARHREALAHL

MmDph1  ESQLRNLGLPFVRLLLSEIFPSKLSLLPEVDVWVQVACPR--LSIDWGSAFPKPLLTPYE
ScDph1  EKNLIAAGKTVVKIILSEVFPQKLAMFDQIDVFVQVACPR--LSIDWGYAFNKPLLTPYE
ScDph2  VKLIKTREKKHYLFVVGKPNVAKLANFEDIDIWCILGCSQSGIIVDQFNEFYKPIITPYE
MmDph2  RKLTEAAGKRSYVLAVGKPTPAKLANFPEMDVFVLLACPLGALAPQPSGGFFRPVLTPCE

MmDph1  AAVALK-DISWQQP-YPMDFYSGSSLGPWTVNYGRDRAPRGLCQPASDKVQQGSRGGSP
ScDph1  ASVLLKKDVMFSEKYYPMDYYEAKGYG---------------------RGETP-
ScDph2  LNLALSEEVTWTGKWVVDFRDAIDEIEQNLGGQDTISASTTSDEPEFDVVRGRYTSTSRP
MmDph2  LEAACNPAWPPGLAPHLTHYAELLPGSPFHVPLPPPESELWDTPDVSLISGELRPPPP-

MmDph1  --------APACESCNCADQKATSPAP-------------
ScDph1  --------KHAIE-------------
ScDph2  LRALTHLELEAADDDDSKQLTTRHTASGAVIKGTVSTSASALQNRSWKGLGSDFDSTEVD
MmDph2  --------WKSSDDTRCSALIPRPQLE---LAESSPAASFLSSRNWQGLEPRLGQTPVK

MmDph1  -------------------------
ScDph1  -------------------------
ScDph2  NTGADIEEGISGVARGYGFDREDAMKKENK
MmDph2  ----EAVRGRRGIAIAYEDEGSS------
```
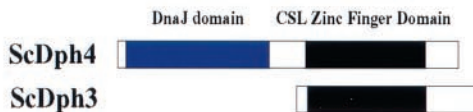
C

DnaJ domain   CSL Zinc Finger Domain

ScDph4

ScDph3

D

CSL Zinc Finger Domain

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                 10        20        30        40        50        60        70
            ....*...|....*...|....*...|....*...|....*...|....*...|....*...|..
consensus  1 DEVEIEDFEFDEEKLSYYYPCP-CGDRGGFQISEEDLEEGED------------VAQCPSCSLVIKVIYD 57
ScDph3     5 DEIEIEDMTFEPENQMFTYPCP-CGDR--FQIYLDDMFEGEK------------VAVCPSCSLMIDVVFD 59
ScDph4    93 DEFSLDDFSFDEDKLEFMMNCPrCQFVGGFHFSESLLDECIDnvdamershsgyqlLTQCSACSLWLKVNFD 164
gi 5824204 78 SIVDLSEFE-ELDNGSYYYPCR-CGDLGGGYVVTEDDLENNRS------------VVPCMGCSLTIQVDYE 133
gi 5306242  4 DDVEIEDMEWNEEIQAYTYPCP-CGDL--FQITKEDLRLGEE------------IANCPSCSLYITVIYN 58
gi 29611912 6 DEVEIEDFEFDEEKDVHYPCP-CGDR--FEIPREMLEMGED------------VAQCPSCSLLIRVIYD 60
gi 29611928 6 DEVEIEDFEYDEEEMYYYPCP-CGDR--FQISKEELIEGEE------------VATCPSCSLIVRVIYD 60
gi 29611779 6 DEVEIEDFQYDEDSETYFCPCP-CGDN--FSITKEELENGEG------------VAMCPGCSLIIKVIYD 60
gi 29611927 5 DEIELEDFTTFDAGTNLYTFCP-CGDR--FEISLEDLQLGED------------VARCPSCSLIVRVIYD 59
gi 7296710 123 SESESEANKGPATMWSYAYDCR-CGGQYLFDGPADDDESPEV------------IVECNECSLVIIVKQA 179
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E

DnaJ-domain

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                 10        20        30        40        50        60        70
            ....*...|....*...|....*...|....*...|....*...|....*...|....*...|..
consensus  1 DYYEILGVPS--NASQEEIKKAYRKLALKYHPDKNP---GDP-----EAEEKFKEINEAYEVLSDPEKRALYDQYG 66
ScDph4     8 THYEILRIPS--DATQDEIKKAYRNRLLNTHPDKLSksiHDT-----VSNVTINKIQDAYKILSNIKTRREYDRLI 76
1FPO_A     2 DYFTLFGLPAryQLDTQALSLRFQDLQRQYHPDKFA---SGSqaeqlAAVQQSATINQAWQTLRHPLMRAEYLLSL 74
gi 1706474 6 TYYDVLGVKP--NATQEEIKKAYRKLALKYHPDKNP---NEG-----EKFKQISQAYEVLSDAKKRELYDKGG 68
gi 18202150 4 DYYKILGIPS--GANEDEIKKAYRKMALKYHPDKNK---EPN------AEEKFKEIAEAYDVLSDPEKKRGLYDQYG 68
gi 5020005 7 DYYEILGVPE--NATEEEIKKAYRKLAREWHPDRNP---DKP-----NAEEKFKEIQEAYSVLSDPEKRRQYDMMR 72
gi 11132549 7 DYYEILGVPR--DATQEEIKKAYRKRLVKEWHPDRHP---ENRk-----EAEQRFKEIQEAYEVLSDPQKRAMYDRFG 73
gi 1346294 21 EFYEILKVDR--KATDSEIKKAYRKLAIKLHPDKNS---HPK------AGEAFKVINRAFEVLSNEEKRSIYDRIG 85
gi 4249377 23 NPYEVLGIPS--NSTDQEIKSAYRRMALRYHPDKNP---DDP-----VAAEMFKEVTFAYEVLSDPENRRLYDTTG 88
gi 14190375 68 SLYEILEIPV--GSTSQEIKSAYRRLARICHPDVAR---NSRdn--sSADDFMKIHAAYCTLSDPEKRAVYDRRT 135
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Dph2-deficient cells migrated on the native gels at a rate intermediate between those of the ADP-ribosylated and non-ADP-ribosylated forms of eEF-2 from the parental cells (Fig. 3C), consistent with the expected net charges on the diphthamide side chains of the various species at neutral pH (Fig. 1A): 0 for His precursor, "intermediate," and diphthine; +1 for diphthamide; and −1 for ADP-ribosyl-diphthamide. In addition, transfection of the respective cDNAs corresponding to the mutated genes completely restored the normal migration rate of the eEF-2 proteins and made them sensitive to the DT-catalyzed change in mobility (Fig. 3C). Surprisingly, the eEF-2 from CHO RPE3b (Dph5; CG-1) cells comigrated with the ADP-ribosylated form of eEF-2 from wild-type CHO cells (Fig. 3C). As expected, transfection of mouse *DPH5* into RPE3b cells made eEF-2 capable of ADP ribosylation, as evidenced by a mobility shift. However, both the ADP-ribosylated and non-ADP-ribosylated forms of eEF-2 migrated faster than the corresponding species from wild-type cells (Fig. 3C). This is most easily explained as due to an unrecognized amino acid substitution in the eEF-2 structural gene that adds a net negative charge.

An interesting observation relevant to the role of diphthamide was that both the nonnative forms of eEF-2, i.e., the mutant, diphthamide-deficient eEF-2 from CG-1, CG-3, and CG-4 and the ADP-ribosylated eEF-2 from wild-type CHO cells, were more reactive with the antibody used in Western blotting, an antibody directed to a peptide sequence at the carboxyl erminus of eEF-2 (Fig. 3C). This was observed with blots from native but not SDS gels, suggesting that the greater reactivity was due to a conformational difference. One possible interpretation is that a conformational change occurs when eEF-2 is ADP ribosylated and that the diphthamide-deficient eEF-2 has a structure that more closely resembles the ADP-ribosylated form rather than the native eEF-2.

Dph1 and Dph2 interact in vivo. The facts that at least three gene products are involved in the first step of diphthamide biosynthesis (Fig. 1A) and that one of them (Dph3) contains only 82 amino acids led us to suggest that these proteins form a catalytic complex (19). To test whether the proteins interact, vectors were constructed to express MmDph1, MmDph2, MmDph5, and HsDph3 with HA or Myc tags at their amino termini. The tagged proteins were each shown to be functional by demonstrating their abilities to complement the respective mutant cells (Table 4) (19). Various combinations of the constructs were transiently transfected into CHO K1 cells, followed by immunoprecipitation of the cell lysates with an agarose-conjugated anti-Myc antibody and immunoblotting with anti-HA antibody. The results showed that MmDph2 could be specifically coimmunoprecipitated with MmDph1 and vice versa (Fig. 3D). In contrast, MmDph5 could not be coimmu-

TABLE 4. Complementation of DT-resistant phenotype of CHO diphthamide-deficient CHO mutants by corresponding *DPH* genes[a]

Cell	Recessive CG	Gene transfected with	*Hyg*[r] clones (DT[s]/total)	Cell source or reference
CHO RPE3b	CG-1	MmDph5	47/48	25
CHO PRE22e	CG-3	MmDph1	0/28	25
		MmDph2	40/44	
CHO PRE33d	CG-3	MmDph1	0/29	25
		MmDph2	35/43	
		MmDph2-HA	19/20	
		MmDph2-Myc	13/14	
CHO PR303	CG-4	MmDph1	40/59	Leppla collection
		MmDph2	0/50	
		MmDph1-HA	18/23	
		MmDph1-Myc	20/26	

[a] Following transfection with the genes indicated, hygromycin-resistant colonies of CHO cells were established by growth for 2 weeks in hygromycin B (500 μg/ml). The cell colonies were marked on the bottom of the dish and then treated with DT (300 ng/ml) for 48 h. The number of toxin-sensitive (i.e., dead) cells versus the total number tested were determined by microscopic observation. Hyg[r], hygromycin resistant; DT[s], DT sensitive; CG, complementation group.

noprecipitated with MmDph2, although MmDph5 was well expressed in the cell lysate (Fig. 3D). Experiments with yeast suggested that Dph3 interacts with Dph1 and Dph2 (10). However, we could not observe potential HsDph3 interactions in these experiments because HsDph3-HA was not expressed at detectable levels (Fig. 3D).

Supporting the structural and functional similarities of diphthamide biosynthesis in lower and higher eukaryotes, ScDph1 and ScDph2 were also shown to interact in yeast. ScDph1 and ScDph2 in vivo coassociation was demonstrated by coimmunoprecipitation experiments like those described above, as well as by two-hybrid analysis. Based on analysis of multiple *DPH2* clones identified from the two-hybrid library, the Dph1-interacting region of Dph2 was localized to residues 50 to 396 (of 534 total) (data not shown). Based on these data, it is likely that Dph1 and Dph2 function in yeast and mammals as a heterodimer or multimer.

DISCUSSION

Mutations affecting the biosynthesis of diphthamide were selected on the basis of resistance to DT that was expressed intracellularly in yeast or added exogenously to cultured mammalian cells. To identify all the genes required for diphthamide biosynthesis, we isolated 847 DT-resistant yeast mutants using both spontaneous and transposon-based mutagenesis approaches. All these mutants fell into the same five complementation groups defined previously by Chen et al. using only 31 DT-resistant mutants (Table 2) (7). The scale of the analysis

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FIG. 2. Sequence alignments of Dph proteins. (A) Phylogenetic analysis of Dph1 and Dph2 from different species. At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*. (B) Sequence alignments of yeast and mouse Dph1 and Dph2 proteins. Identical residues are shaded in black, and similar residues are shaded in gray. Dashes represent gaps in the aligned sequences. (C) Schematic representation of domain arrangements of ScDph3 and ScDph4 proteins. (D) The consensus CSL zinc finger domain sequence from the NCBI conserved-domain database aligned with the amino-terminal region of ScDph3 (residues 5 to 59), the carboxyl-terminal region of ScDph4 (residues 93 to 164), and other related proteins. In the alignment, identical residues are in blue and the four cysteine residues and the highly conserved CSL zinc finger motif are in red. (E) The consensus Dna-J sequence from the NCBI conserved-domain database aligned with the N-terminal region of ScDph4 (residues 8 to 76) and other related proteins. In the alignment, identical residues are in blue and the J-domain signature HPD motif is in red.
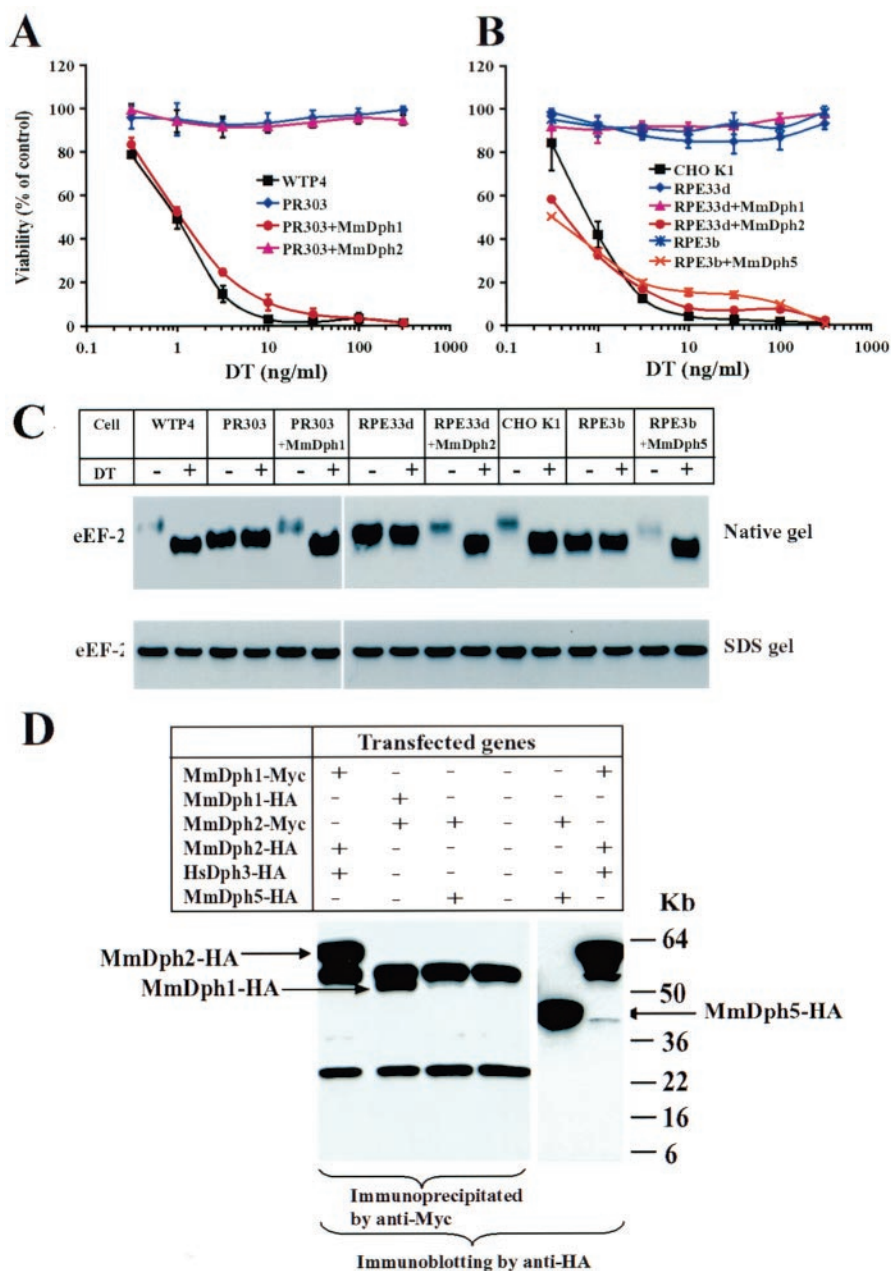
FIG. 3. Complementation of the diphthamide-deficient CHO mutant cells by transfection with the respective mouse *DPH* genes. (A) Expression of MmDph1 in CG-4 PR303 cells complements their DT-resistant phenotype. Representative purified clones of PR303 cells transfected with mouse *DPH1* or mouse *DPH2* were incubated with various concentrations of DT for 48 h, and MTT was added to determine cell viability. Optical density readings were normalized to values for cells that received no toxin to calculate percent viability. (B) Expression of MmDph2 in CG-3 RPE33d cells and MmDph5 in CG-1 RPE3b cells complements their DT-resistant phenotypes. Cytotoxicity of DT to representative clones of RPE33d cells transfected with mouse *DPH1* or mouse *DPH2* and to a representative clone of RPE3b cells transfected with mouse *DPH5* were determined as in panel A. (C) The CHO cell lysates were incubated with or without DT at room temperature for 30 min and then analyzed by either native PAGE (top) or SDS-PAGE (bottom), followed by Western blotting using an antibody against a linear peptide of the carboxyl terminus of eEF-2. +, present; −, absent. (D) Dph1 and Dph2 can physically interact in vivo. Combinations of HA- or Myc-tagged MmDph1, MmDph2, HsDph3, and MmDph5 expression plasmids as indicated were transiently transfected into CHO K1 cells for 24 h. Cell lysates were prepared and immunoprecipitated using an agarose-conjugated anti-Myc antibody, followed by Western blotting using an anti-HA antibody. Expression of MmDph5-HA in MmDph2-Myc- and MmDph5-HA-transfected cells was evidenced by direct Western blot analysis of the cell lysate using the anti-HA antibody. The two bands common in lanes 1 to 4 are to the heavy and light immunoglobulin G chains.

and the frequency of the mutants strongly suggest that the five loci defined by *DPH1*- to -5 encode all the proteins required for the first and second steps of diphthamide biosynthesis (Fig. 1A). The *DPH2* and *DPH5* genes have been characterized previously. In this work, the other three yeast genes, *DPH1*, *DPH3*, and *DPH4*, were identified. We also identified the mammalian *DPH* homologues in diphthamide-deficient CHO mutants of three complementation groups, CG-1 (*dph5*), CG-3

(*dph2*), and CG-4 (*dph1*). Our previous work identified the gene altered in CHO CG-2 (*dph3*). All these *DPH* genes have clear homologues in many organisms and likely function in similar manners, as demonstrated in yeast and CHO cells in this study. The *DPH* genes in yeast and mammalian cells are summarized in Table 3 and discussed below.

Dph1. We identified ORF *YIL103w* as the gene mutated in yeast *dph1* mutants. This ORF encodes a 425-residue polypeptide having 49% identity with mouse Dph1 that we were able to show specifically complements the diphthamide biosynthetic defect in CHO CG-4 cells. While this is the first demonstration that *DPH1* is required for diphthamide modification in higher eukaryotes, the mouse and human *DPH1* genes were previously identified and characterized as *OVCA1* (31) and *DPH2L1* (28) for very different reasons. Intriguingly, *DPH1* of mouse and human origins has been recently identified as a tumor suppressor gene. Human *DPH1* is located in a highly conserved region on chromosome 17p13.3, which shows frequent loss of heterozygosity (deletion of one allele) in ovarian and breast carcinomas (2, 28, 29, 31). Western blot analysis of extracts prepared from ovarian and breast cancer cells revealed reduced expression of HsDph1 compared with that from normal epithelial cells from these tissues (2), suggesting that haploinsufficiency of this gene accounts for tumorigenesis. Forced overexpression of human *DPH1* caused suppression of cell proliferation and colony formation by ovarian cancer cell lines (2). Furthermore, knockout of one allele of *DPH1* in mice leads to increased tumor development, whereas loss of both alleles results in embryonic lethality (5). It appears probable that the effects of MmDph1 deficiency on cell proliferation, embryonic development, and tumorigenesis result from the decrease in, or absence of, diphthamide. The generation of mice lacking other genes in the diphthamide biosynthesis pathway will help to clarify this issue.

Dph2. The yeast *DPH2* gene encodes a 534-residue polypeptide (23) with modest similarity to Dph1 (16% identity). ScDph2 is 22% identical to the predicted product encoded by mouse *DPH2*, which we showed complements the diphthamide biosynthetic defect of the CHO CG-3 mutants. This result unequivocally establishes that Dph2 has a role in diphthamide biosynthesis in higher eukaryotes and also links Dph2 to CG-3. We showed that Dph1 and Dph2 physically interact in vivo in both yeast and mammalian cells. This suggests that these proteins function as a heterodimer or part of heteromultimeric complex. Despite their primary structural similarity, mutation in either *DPH1* or *DPH2* completely blocks diphthamide formation in both yeast and CHO cells, demonstrating that the two proteins are functionally independent in diphthamide biosynthesis. Recently, these two proteins were shown in a global analysis of protein localization to be in the cytoplasm (14), where diphthamide modification of eEF-2 is expected to occur.

Dph3. We identified ORF *YBL071w-A* as the gene mutated in yeast *dph3* mutants. Our previous study had identified *DESR1* as the gene mutated in the CHO CG-2 mutant cells and had shown that the yeast homologue ORF *YBL071w-A* is required for diphthamide biosynthesis in yeast (19). The new result associates Dph3 with DESR1. HsDph3 and ScDph3 are small proteins of only 82 residues which possess a high percentage of negatively charged residues. The sequences of Dph3 from various species are highly conserved in residues 1 to 60

(Fig. 2D) but differ at the carboxyl termini (19). A search of the National Center for Biotechnology Information (NCBI) conserved domain database (20) identified the conserved amino-terminal region as a typical CSL zinc finger domain (this domain is named after the conserved three-residue motif containing the final cysteine) (Fig. 2C and D). The four cysteines in CSL domains are thought to chelate zinc, suggesting that the function of Dph3 and its homologues may depend on their zinc-binding abilities. Sequence analysis of the *dph3-1* allele in the original *dph3* strain isolated by Chen et al. (7) revealed a G-to-A mutation which would result in the substitution of a tyrosine for the cysteine in the CSL motif of the protein (Fig. 2D and data not shown). This result shows that the CSL zinc motif is crucial to the function of Dph3. The global localization analysis mentioned above showed that Dph3 localizes in both the cytoplasm and the nucleus (14).

Yeast *DPH3* has also been characterized as *KTI11*—a gene conferring sensitivity to zymocin, a toxin secreted by the dairy yeast *K. lactis* (11, 19). Using tandem affinity purification analysis, a recent study showed that the Kti11 protein associates with Dph1, Dph2, and eEF-2 and also with the subunits of the yeast transcriptional Elongator core complex, Elp1, Elp2, and Elp3, that are required for zymocin sensitivity (10). This raised the possibility that the Elongator complex might be involved in diphthamide biosynthesis. Indeed, *dph1* and *dph2* mutants are moderately resistant to zymocin (10). However, although there does appear to be a link between diphthamide biosynthesis and the Elongator complex, it is not reciprocal, as we demonstrated that *elp1*, *elp2*, and *elp3* yeast mutants are not defective in diphthamide biosynthesis and thus are sensitive to DT treatment (data not shown).

The association of Dph3 with Dph1 and Dph2 mentioned above suggests the existence of a catalytic protein complex, as was implied by the participation of three genes in the first step of diphthamide synthesis. Interestingly, eEF-2 also copurified with the Dph1, Dph2, and Dph3 complex, along with two ribosomal proteins (10). However, the same tandem affinity purification analysis did not detect Dph4 as a Dph3 interactor, suggesting that Dph4 may function separately or indirectly in diphthamide biosynthesis (discussed further below).

Dph3 is likely to play multiple roles in cellular physiology. Yeast mutants lacking *DPH1*, *DPH2*, *DPH4*, or *DPH5* exhibit relatively subtle physiological effects and are largely indistinguishable from wild-type controls (with the exception of resistance to DT) (T. Milne and G. R. Fink, unpublished data). However, in contrast, *dph3* mutants have pleiotropic growth defects, including slow growth and drug and temperature hypersensitivity, in addition to DT resistance (11; Milne and Fink, unpublished).

Dph4. We identified *DPH4* as ORF *YJR097w*, encoding a 172-residue DnaJ-like protein. This *DPH* gene is the only one of the five for which no corresponding CHO mutant has been obtained. An NCBI conserved-domain database search demonstrated that the first half of the ScDph4 protein (residues 8 to 76) is a typical J domain (Fig. 2C and E) that contains the J-domain signature HPD motif (27). Over 100 DnaJ proteins have been identified (12). The roles most clearly defined thus far for DnaJ-like proteins are as cochaperones aiding HSP70 proteins in the folding of the newly synthesized proteins (12). Therefore, we assume that Dph4 may function in diphthamide

biosynthesis by ensuring the proper folding of one or more of the other Dph proteins. Another interesting feature of the Dph4 structure is that the second half of the protein (residues 93 to 164) consists of a typical CSL zinc finger domain, which aligns well with that of Dph3 (Fig. 2D), suggesting that the zinc-binding ability could be essential to its function. The presence of a CSL motif differentiates Dph4 from many other DnaJ-like proteins and may also be indicative of a specialized role. Although we did not directly identify any mammalian homologs of *DPH4* in our genetic screens, searching of the GenBank databases showed that the highly conserved 193-residue mouse DnaJ-like protein, MmDjC7 (17), and its 148-residue splicing isoform are probable Dph4 homologs (Table 3). Both have the same domain arrangement as ScDph4—an amino-terminal DnaJ domain followed by a carboxyl-terminal CSL zinc finger domain (20). Interestingly, MmDjC7 has been shown to be ubiquitously expressed and to function as a co-chaperone in stimulation of the ATPase activities of several HSP70 proteins, including BiP, Hsc70, and DnaK (17). Like ScDph3, ScDph4 was shown in yeast to localize in both the cytoplasm and the nucleus (14).

Dph5. Yeast *DPH5* was previously identified as ORF *YLR172c*, encoding a 300-residue AdoMet-dependent methyltransferase (22). Dph5 acts in the second biosynthetic step, trimethylation of the diphthamide intermediate to produce diphthine, the step impaired in CHO CG-1 mutants. As expected, ScDph5 is localized in the yeast cytoplasm (14). ScDph5 is highly homologous with its mammalian homologue; ScDph5 shows 55 and 50% identity with MmDph5 and HsDph5, respectively. Dph5 proteins from different species are members of a large family of methyltransferases, but there is no evidence of functional redundancy for diphthamide biosynthesis in either yeast or CHO cells. As expected, MmDph5 can restore the DT-resistant phenotypic change of the CG-1 diphthamide-deficient CHO mutant RPE3b (Table 4), a well-characterized CHO mutant impaired in the trimethylation step of diphthamide biosynthesis, confirming the link of Dph5 to CG-1.

Biosynthesis and role of diphthamide. The work described here provides a complete description of the five gene products that are required for the biosynthesis of diphthamide. With this information in hand, it is now possible to design structural and biochemical analyses to elucidate the role of each protein in the synthetic process. It appears probable that Dph1, Dph2, and Dph3 form a multimeric complex that catalyzes the transfer of the four-carbon chain onto the eEF-2 His[699] side chain. The Dph4 protein may play a role in assembling this complex. The inability to get in vitro complementation by mixing cell lysates of the CHO mutants of CG-2 and CG-3 (26) suggests that any multimeric complex is not readily dissociable, as might be expected if Dph4 acts as an assembly chaperone. It is interesting that the trimethylation of diphthine requires Dph5 and cannot be performed by the many other methylases in mammalian cells. It remains to be determined whether Dph5 catalyzes the transfer of all three methyl groups or only some, with the others added by a less specific methyltransferase.

Although diphthamide is the molecular target for DT and ETA, its biological significance remains elusive. The evolutionary conservation of the complex diphthamide biosynthesis pathway throughout eukaryotes implies a key biological role of diphthamide. This role could be either structural or regulatory. In support of a structural role, systematic mutagenesis of eEF-2 His[699] to each of the remaining 19 amino acids resulted in non-ADP-ribosylatable eEF-2 variants that were either inactive (6 of 19) or temperature sensitive for growth (13 of 19) (16). The six nonfunctional alleles underscore the key function of this residue in eEF-2, while the temperature-sensitive alleles suggest that diphthamide may play a role in overall eEF-2 structure or stability. In fact, we observed that the non-diphthamide-modified form of eEF-2 in various CHO *dph* mutants was bound more readily by anti-eEF-2 antisera than the diphthamide-modified form (Fig. 3C). It is known that eEF-2 undergoes conformational changes during its action on the ribosome (15), so alterations that affect its flexibility could have large effects on its function.

Alternatively, diphthamide may serve as the site of a regulatory modification of eEF-2. Mono-ADP-ribosylation is commonly used in bacterial systems for posttranslational regulation (21). There have been suggestions of similar regulatory mechanisms in eukaryotic systems (8). In fact, the existence of a cellular ADP ribosyltransferase with the same mechanism of action as DT has been reported (18). Furthermore, the discovery in this work that *DPH1* is a recently characterized tumor suppressor gene (5) suggests a potential role for diphthamide in the control of cell growth and tumorigenesis.

In this study, we cloned and characterized three previously unidentified *DPH* genes and the genes impaired in CG-1, CG-3, and CG-4 CHO mutants. Further biochemical and in vivo targeting analyses of these genes will help to define their precise roles in diphthamide biosynthesis and elucidate the mysterious role of diphthamide in cellular physiology.

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