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The diphthamide modification on elongation factor-2 renders mammalian cells resistant to ricin

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Summary

Diphthamide is a post-translational derivative of histidine in protein synthesis elongation factor-2 (eEF-2) that is present in all eukaryotes with no known normal physiological role. Five proteins Dph1-Dph5 are required for the biosynthesis of diphthamide. Chinese hamster ovary (CHO) cells mutated in the biosynthetic genes lack diphthamide and are resistant to bacterial toxins such as diphtheria toxin. We found that diphthamide-deficient cultured cells were 3-fold more sensitive than their parental cells towards ricin, a ribosome-inactivating protein (RIP). RIPs bind to ribosomes at the same site as eEF-2 and cleave the large ribosomal RNA, inhibiting translation and causing cell death. We hypothesized that one role of diphthamide may be to protect ribosomes, and therefore all eukaryotic life forms, from RIPs, which are widely distributed in nature. A protective role of diphthamide against ricin was further demonstrated by complementation where *dph* mutant CHO cells transfected with the corresponding *DPH* gene acquired increased resistance to ricin in comparison with the control transfected cells, and resembled the parental CHO cells in their response to the toxin. These data show that the presence of diphthamide in eEF-2 provides protection against ricin and suggest the hypothesis that diphthamide may have evolved to provide protection against RIPs.

Introduction

Diphthamide, a unique amino acid, is a post-translational derivative of histidine that is present only in protein synthesis elongation factor 2 of eukaryotes (eEF-2) (Bodley *et al.*, 1984; Moehring *et al.*, 1980; Van Ness *et al.*, 1980). Diphthamide is the target of the bacterial toxins diphtheria toxin (DT) and *Pseudomonas* exotoxin A (ETA) (Liu and Leppla, 2003; Oppenheimer and Bodley, 1981) and of fusion proteins derived from them that are being developed as anticancer agents (Liu *et al.*, 2003; Pastan *et al.*, 2007). These toxins transfer ADP-ribose from NAD to diphthamide (Fig. 1). The ADP-ribosylated eEF-2 can no longer perform its normal function in the translational process and intoxicated cells that are unable to synthesize proteins eventually die. DT and ETA are single polypeptides which bind the receptors heparin-binding epidermal growth factor-like growth factor precursor and low-density lipoprotein receptor-related protein-1, respectively (FitzGerald *et al.*, 1995; Naglich *et al.*, 1992).

Our laboratory has made extensive use of a fusion protein designated FP59, which contains the ADP-ribosylating catalytic domain of ETA fused to a portion of anthrax toxin. Anthrax toxin consists of the single cell-binding moiety protective antigen (PA) and two alternate catalytic subunits known as lethal factor (LF) and edema factor (EF) (Leppla, 2006). PA is the

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central component which is essential for translocation of LF and EF into the cytosol. PA uses tumor endothelium marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) as receptors to gain entry into the cells (Leppla, 2006). In the fusion protein FP59, LF amino acids 1−254 are fused to the catalytic domain of ETA such that administration with PA causes delivery of the ADP-ribosylating activity to the cytosol (Arora *et al.*, 1992). Because PA receptors are widely distributed, the combination of PA and FP59 is highly toxic to nearly all cell types. The three ADP-ribosylating toxins (DT, ETA, PA+FP59) have diphthamide as their sole target, as shown by the facile selection of somatic mutant cells cross-resistant to all three toxins, and the demonstration that these have eEF-2 lacking diphthamide (Liu *et al.*, 2006; Moehring *et al.*, 1980; Moehring and Moehring, 1979).

Diphthamide biosynthesis requires five proteins, Dph1 to Dph5, which work cooperatively and sequentially to assemble the side chain on the precursor His^{715} (His^{699} in yeast) in eEF-2, leading to diphthamide (Fig. 1) (Chen and Bodley, 1988;Liu *et al.*, 2004;Moehring *et al.*, 1984). The first step is transfer of a 3-amino-3-carboxypropyl group to the imidazole C-2 of the precursor histidine using S-adenosyl methionine (AdoMet) as donor. This step requires a coordinated action by Dph-1 to −4 (Liu *et al.*, 2004;Liu and Leppla, 2003). The second step, catalyzed by Dph5, is trimethylation of the 3-amino-3-carboxypropyl group to produce diphthine (Mattheakis *et al.*, 1992). In the final step, amidation of carboxyl group, by a yet unknown amidase, yields diphthamide. Mutations have been found in yeast and somatic cells that fail to perform many of these steps.

Because diphthamide-deficient somatic cell and yeast mutants are viable (Chen *et al.*, 1985; Liu *et al.*, 2004), it is evident that eEF-2 lacking diphthamide is still able to perform its basic role in protein synthesis. The question then arises as to why this complex, apparently dispensable post-translational modification has evolved and been maintained in all eukaryotes, and also in the archaea (Pappenheimer, Jr. *et al.*, 1983). Many studies have been done that attempt to decipher the role of diphthamide in eEF-2, but they are not conclusive. Site directed mutagenesis studies of the precursor histidine residue are consistent in showing that eEF-2 lacking diphthamide at His^{699} (in yeast) or His^{715} (in mammals) retains activity in protein synthesis while failing to be a substrate for DT (Ivankovic *et al.*, 2006; Kimata and Kohno, 1994; Phan *et al.*, 1993). The first direct evidence of a beneficial action was the recent demonstration that diphthamide increases translational accuracy, because yeast strains having diphthamide-deficient eEF-2 showed increased −1 frameshifting (Ortiz *et al.*, 2006).

The region on the ribosome at which eEF-2 acts overlaps with the site targeted by "ribosome inactivating proteins" (RIPs). RIPs comprise a family of toxic proteins with representatives in fungi (alpha-sarcin) and pathogenic bacteria (Shiga toxin), and a large number of both single and two-chain proteins in plants, where they may act to discourage consumption by animals (Barbieri *et al.*, 1993; Perentesis *et al.*, 1992). RIPs act as N-glycosidases that inactivate 60 S ribosomal subunits by hydrolyzing the N-glycosidic bond of an adenosine residue (A4324) in the "ricin-sarcin loop" of 28 S rRNA. Ricin, a toxic protein from the seeds of *Ricinus communis*, is representative of two-chain RIPs which have a receptor-binding chain that facilitates cell entry and high potency (Olsnes and Kozlov, 2001). In contrast, a larger and diverse set of RIPs are single-chain proteins (e.g., saporin) which lack a receptor-binding domain and rely on non-specific mechanisms to gain entry to cells (Perentesis *et al.*, 1992).

Previous studies *in vitro* have shown that ricin-induced inactivation of ribosomes can be overcome by adding higher concentrations of eEF-2 (Brigotti *et al.*, 1989; Fernandez-Puentes *et al.*, 1976; Holmberg and Nygard, 1994). Binding of eEF-2 in the presence of non-hydrolyzable GTP analogue GuoPP[CH_2]P completely protected ribosomes from ricin (Holmberg and Nygard, 1994). However, there appear to be no studies in which the binding of eEF-2 lacking diphthamide to ribosomes was measured. In the present study, we analyzed

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the comparative sensitivity to ricin of the various CHO cells having wild type or diphthamide-deficient eEF-2 and found that diphthamide protects against ricin. Based on this result, we speculate that diphthamide may have evolved to protect against ribosome inactivating proteins.

Results

CHO cells having *DPH3* inactivated show increased sensitivity towards ricin

To identify mammalian genes required for anthrax toxin action, we previously performed retroviral insertional mutagenesis in CHO cells (Liu and Leppla, 2003). The mutagenized CHO cell population was selected with PA plus FP59 and many toxin resistant mutants were obtained. These mutant cells were further screened for their sensitivity against various toxins including DT, ETA and ricin. Most of the mutant clones were resistant only to PA plus FP59 and were determined to be PA receptor-deficient mutants (data not shown), whereas two of the mutants, PR72 and PR201, showed a different phenotype. PR72 and PR201 were resistant not only to PA plus FP59 (Fig. 2a), but also were completely resistant to DT and ETA (data not shown). These cells were shown to be mutated in a gene now designated *DPH3* (Liu *et al.*, 2006) that is required for diphthamide biosynthesis (Fig. 1). Using PR72 as representative of the two *dph3* mutant cell lines, we confirmed the inability of eEF-2 to be ADP-ribosylated by ADP ribosylating toxin, FP59 (Fig. 2a, inset). Surprisingly, PR72 and PR201 showed increased sensitivity to ricin; the EC_{50} values for PR72 and PR201 cells were reduced by 3-fold as compared to their parental cells, WTP4 (Fig. 2b, and Table 1). Since both PR72 and PR201 cells lack diphthamide on eEF-2, we considered whether the absence of this unique post-translational modification might be responsible for increased sensitivity to ricin.

CHO cells with other *DPH* genes inactivated are also hypersensitive towards ricin

Recently, the *DPH3* gene has been shown to be required for modifying certain tRNAs (Huang *et al.*, 2005), and therefore the Dph3 defect in PR72 and PR201 cells could theoretically have altered sensitivity to ricin in other ways. Therefore we analyzed additional CHO mutant cells lacking diphthamide because of defects in other *DPH* genes. RPE22e and RPE3b cells were generated from their parental CHO-K1 cells by chemical mutagenesis and selection for resistance to DT (Moehring *et al.*, 1980; Moehring *et al.*, 1984). RPE3b cells do not have functional Dph5 while RPE22e cells lack functional Dph2 protein (Liu *et al.*, 2004; Moehring *et al.*, 1984). RPE3b and RPE22e cells showed complete resistance towards the ADP-ribosylating toxin FP59 in combination with PA, while CHO-K1 cells were highly sensitive (Fig. 2c), confirming that CHO-K1 cells have wild type eEF-2, while RPE3b and RPE22e cells have eEF-2 lacking diphthamide. *In vitro* ADP-ribosylation assays further confirmed the presence of ADP-ribosylatable eEF-2 specific to CHO-K1 cells (Fig. 2c, inset). In cytotoxicity experiments, RPE3b and RPE22e cells exhibited significant hypersensitivity towards ricin as compared to their parental cell line, CHO-K1 (Fig. 2d). The EC_{50} was 3-fold lower for both RPE22e and RPE3b as compared to CHO-K1 cells (Table 1).

Complementation of diphthamide biosynthesis in diphthamide-deficient cells restores their resistance to ricin

To further verify that the hypersensitivity of the mutant cells towards ricin is due to the absence of the diphthamide modification on eEF-2, the cells were transfected with the corresponding *DPH* genes to complement diphthamide biosynthesis. The empty expression plasmid or a plasmid harboring an unrelated gene was also transfected into each cell type to be used for comparative analysis. Many independent stable cell lines were generated by expansion of individual clones from each transfection. RPE3b cells were transfected with either pIRES-*DPH5* or empty vector (pIRES-hyg-2). *DPH5* encodes a 300-residue methyl transferase (Mattheakis *et al.*, 1992). Expression of Dph5 in RPE3b cells made them sensitive to PA plus FP59 while control transfected cells were still resistant, as expected (Fig. 3a).

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Complementation was further confirmed by an *in vitro* ADP-ribosylation assay (Fig. 3a, inset). For toxicity assays with ricin, a number of *DPH5* and control vector transfected cell lines were assayed to assure that any changes observed were not restricted to single cell line. All the *DPH5* transfectants were more resistant than any of the control transfectants (Fig. 3b). The average EC_{50} for *DPH5* transfected RPE3b cells was 4.7-fold higher than for vector transfected RPE3b cells (Table 2).

RPE22e cells, which lack functional Dph2, were complemented with the wild type gene. *DPH2* encodes a 534-residue protein having an unknown role in the first step of diphthamide biosynthesis (Mattheakis *et al.*, 1993). As expected, cells transfecting with *DPH2* became sensitive to PA plus FP59 in combination with PA while control transfected cells remained resistant (Fig. 3c). Complementation also restored the activity of eEF-2 to ADP-ribosylation by FP59 (Fig. 3c, inset). Assays for sensitivity to ricin yielded results paralleling those for Dph5, in that restoration of diphthamide biosynthesis led to increased resistance to ricin (Fig. 3d). The average EC_{50} for *DPH2* transfected cells was 3.3-fold higher than for the control transfected cells (Table 2).

Finally, PR72 and PR201 cells, described above as lacking functional Dph3, were transfected with pIRES-*DPH3* or the same vector carrying an unrelated gene, *VPS11*. Cells stably transfected with *DPH3* had the expected sensitivity to PA plus FP59 confirming the expression of Dph3 (data not shown). Cytotoxicity assays with ricin showed that PR72 and PR201 cells transfected with *DPH3* had increased resistance as compared to the control transfected cells (Fig. 3e). The average EC_{50} of *DPH3* transfected cells increased 3.2-fold in comparison with control transfected cells (Table 2). These results for Dph3 are consistent with those for Dph2 and Dph5 in showing that the presence of diphthamide in eEF-2 increases the resistance of CHO cells to ricin by about 3-fold.

Discussion

Diphthamide, the post-translational side chain modification to His^{715} in mammalian eEF-2 (His^{699} in yeast) is unique in several respects. This modification occurs only on eEF-2, it is conserved in all eukaryotes and apparently in archaea, and it serves as the single target for several ADP-ribosylating toxins that are key virulence factors for the bacteria that secrete them. The retention by eukaryotes of a unique target that makes them susceptible to attack by pathogenic bacteria clearly implies that diphthamide serves some important function in normal cellular physiology. However, the role of diphthamide in cellular physiology is not defined yet. The viability of somatic cell (CHO) mutants like those used here shows that eEF-2 lacking diphthamide can function in protein synthesis at a rate and with a degree of accuracy that are sufficient to maintain life. It follows that any important functions of eEF-2 that depend on its containing diphthamide must be relatively subtle, and are likely to become evident only under special circumstances (e.g., stress) or in the context of a multicellular organism.

Disruption of *DPH1* and *DPH3* in mice showed that diphthamide or its biosynthetic genes play important roles during early stages of development. Thus, *DPH1* knockout mice died at an early age (Chen and Behringer, 2004) while *DPH3* knockout mice showed embryonic lethality (Liu *et al.*, 2006). Later work showed that Dph3 is also required for tRNA modification (Huang *et al.*, 2005), which could explain the more severe phenotype of this knockout mouse. However, the early death of the *DPH1*-deficient mice, where the deficit appears restricted to diphthamide biosynthesis, suggests that even modest changes to the accuracy or efficiency of the protein synthetic process can have profound effects in the whole animal at certain stage. However, the evidence presented here suggests an alternative hypothesis regarding the role of diphthamide. Several CHO cell lines have been isolated which lack one of the functional Dph proteins. We noted that two independent CHO cells mutants, PR72 and PR201, lacking functional Dph3 and

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therefore diphthamide (Liu and Leppla, 2003), showed increased sensitivity to ricin. Because several previous studies showed that binding of eEF-2 to ribosomes prevents access by ricin to the susceptible loop in the rRNA (Brigotti *et al.*, 1989; Fernandez-Puentes *et al.*, 1976) we formed the simple hypothesis that diphthamide-containing eEF-2 might block ricin access to ribosomes and thereby protect intact cells against ricin. To test the hypothesis, other diphthamide-deficient CHO mutant cells were analyzed. We selected RPE3b and RPE22 cells which have been well characterized for their DT resistance phenotype and lack functional Dph5 and Dph2, respectively (Liu *et al.*, 2004; Moehring *et al.*, 1980; Moehring *et al.*, 1984). These cells were also found to be hypersensitive to ricin and showed 3-fold lower EC_{50} as compared to their parental cells CHO-K1. To further confirm that observed hypersensitivity is a result from the diphthamide deficiency, not some other unrecognized mutations in diphthamide-deficient cells, these cells were complemented for diphthamide biosynthesis.

Cells were transfected with the plasmid pIRES-hyg2 harboring the specific *DPH* gene or control vector. RPE3b and RPE22e cells transfected with *DPH5* and *DPH2*, respectively, regained the parental degree of resistance to ricin. In another experiment, expression of *DPH3* in PR72 and PR201 cells restored the parental cell resistance towards ricin. In each case, the differences in sensitivity were about 3-fold, regardless of the parental line. All these results clearly indicate that diphthamide-deficient cells show increased sensitivity to ricin as compared to the corresponding diphthamide-containing cells.

Studies cited above showed that ricin and eEF-2 have overlapped binding sites on the ribosome. We speculate that eEF-2 containing the diphthamide modification may have greater affinity for the ribosome, and therefore may more effectively occlude the site targeted by ricin and other RIPs. Given the wide distribution of RIPs in nature, and especially of the single-chain RIPs in plants, it is reasonable to assume that they impose a modest but continuous selective pressure on other organisms, and especially on those species like eukaryotes that eat plants (Barbieri *et al.*, 1993; Perentesis *et al.*, 1992). Although the single-chain RIPs do not enter eukaryotic cells efficiently, their high concentration in some plants suggests that they are toxic under some circumstances (Peumans *et al.*, 2001). The cytotoxicity of the single-chain pokeweed antiviral protein toward virally-infected cells demonstrates the potential of single-chain RIPs to damage cells (Parikh and Tumer, 2004). This ability would seem to be sufficient to select for heritable changes to the protein synthetic machinery that protect it from inactivation. The questions arises whether creation of the diphthamide residue provided such a selective advantage at some stage during evolution, and also whether the 3-fold level of resistance observed here is sufficient to drive and maintain this post-translational change. The organisms competing by modifying these sites may not have resembled those now in existence, so reconstruction of the early evolutionary events must remain speculative. It does appear reasonable to argue that a 3-fold selective advantage is sufficient to assure gene retention when multiplied over many generations in which the selective pressure from RIPs is constant. Some evidence for (or against) the hypothesis that diphthamide evolved in response to pressure from RIPs may eventually be obtained by careful analyses of the extensive genomic DNA sequence information now becoming available, but this is beyond the scope of this study.

Experimental Procedures

CHO cell lines and cell culture

All the cell lines used in this study are derivatives of CHO cells. CHO-K1, RPE3b and RPE22e were derived by Thomas and Joan Moehring and were provided by Gary Ward (University of Vermont), as described previously (Liu *et al.*, 2004). All cell lines were grown in α-minimal essential medium (MEM) supplemented with 8% fetal bovine serum, 2 mM glutamine, 50 μg/ml gentamicin, and 25 mM HEPES.

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Transfection

All the *DPH* constructs used for the transfection were described earlier (Liu *et al.*, 2004; Liu and Leppla, 2003). All *DPH* genes were from mouse origin. The expression plasmids were transfected into CHO cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and selected by growing them in medium with hygromycin- B (500 μg/ml) for 2 weeks. Individual clones were picked and expanded.

Cytotoxicity assays

PA and FP59 were produced as described previously (Arora *et al.*, 1992; Liu *et al.*, 2007). Ricin was purchased from Boehringer Mannheim. Cytotoxicity assays were done as described previously (Liu and Leppla, 2003). In brief, the cells were sub-cultured in 96-well plate one day prior to experiment. For cytotoxicity assays, cells were incubated with various concentrations of FP59 in combination with a fixed amount (500 ng/ml) of PA for 48 h. At the end of incubation, 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) dissolved in MEM was used to measure cell viability. A_{540} values obtained for cells which were not treated with toxin were considered as 100 % viability. Ricin toxicity assays were conducted in the same way.

ADP-ribosylation assay

The assay for ADP-ribosylation of eEF-2 in CHO cell extracts was performed as described previously with some modification (Liu *et al.*, 2004). Biotin-NAD was used as source of ADP-ribose for ADP-ribosylation and transfer of biotin-ADP-ribose to eEF-2 in the presence of toxin was detected by Western blotting using streptavidin-conjugates. In brief, cells were lysed in RIPA buffer containing protease inhibitors and cell lysate (5 μl containing 50 μg protein) was mixed with 500 ng of FP59 in ADP-ribosylation buffer (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 50 mM DTT) with 5 μM 6-Biotin-17-NAD (Trevigen) followed by incubation at 25°C for 30 min. Samples were then mixed with SDS sample buffer, boiled for 5 min and run on 4–25 % SDS-PAGE gels (Invitrogen). The proteins were transferred to nitrocellulose membranes using the iBlot system (Invitrogen) and western blotting was performed using streptavidin-IR conjugate (Rockland Immunochemicals, Gilbertsville, PA) and scanned on an Odyssey Infrared Imager (LICOR Biosciences, Lincoln, NE).

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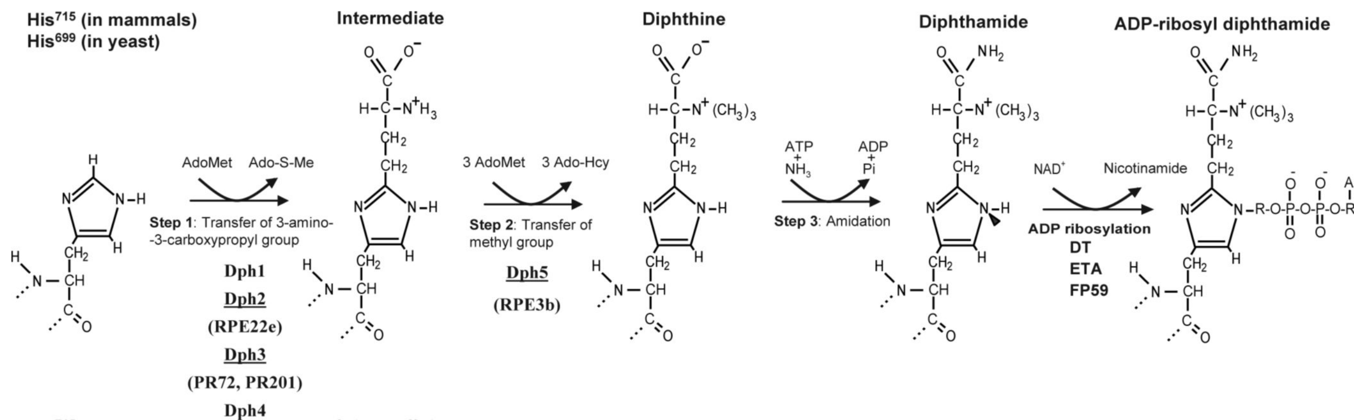

Fig. 1. Diphthamide biosynthetic pathway and ADP-ribosylation

Dph1 to Dph5 are required for the biosynthesis of diphthamide. Dph1 to Dph4 are involved in the first step, the transfer of 3-amino-3-carboxypropyl to the His^{715} (His^{699} in yeast) of eEF-2. Dph5 acts as methyl transferase in the next step, yielding diphthine. Amidation of diphthine is the last step of diphthamide biosynthesis. Arrowhead (N-1 of the histidine imidazole ring of diphthamide) indicates the site of ADP-ribosylation by ADP-ribosylating toxins. AdoMet, S-adenosyl methionine; Ado-S-Me, methylthioadenosine; Ado-Hcy, S-adenosylhomocysteine. In ADP-ribosyl diphthamide, A, adenine moiety; R, ribosyl moiety. The CHO cell mutants lacking corresponding functional Dph proteins used in this study are shown in parentheses.

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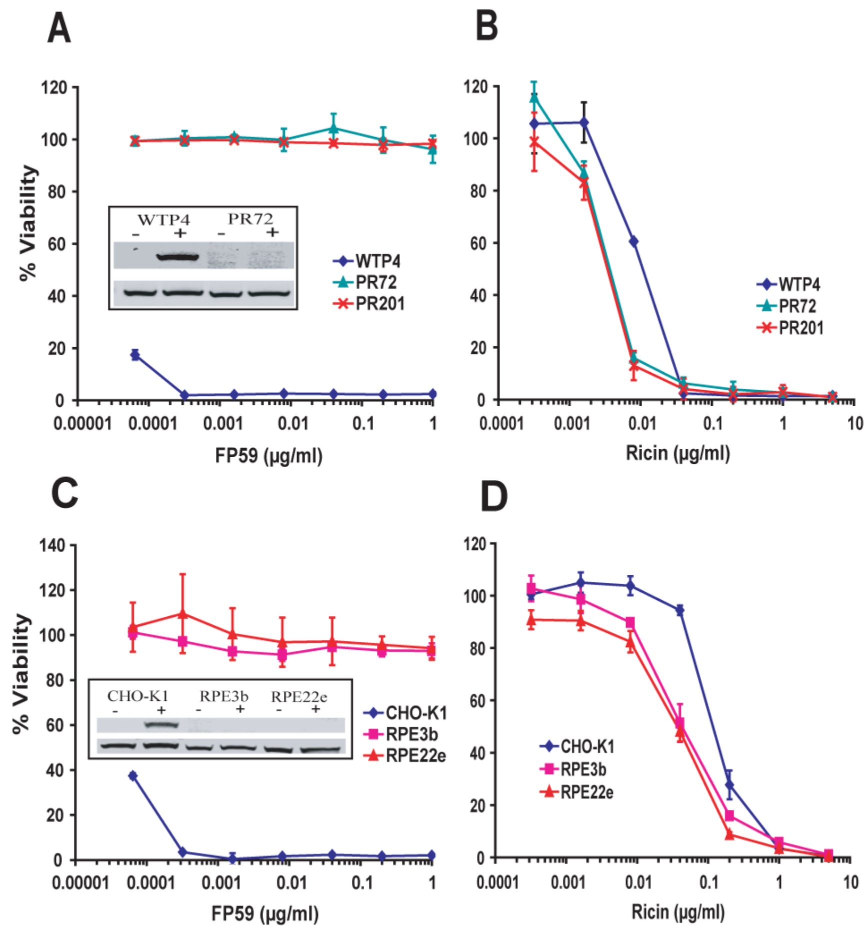

Fig. 2. Toxin sensitivity of diphthamide-deficient CHO mutant cells compared to their parental cell lines

Cells were incubated with the indicated concentrations of FP59 + 500 ng/ml PA (a, c) or with ricin (b, d) for 48 h and MTT assay was done to measure cell viability. The A_{540} values obtained for cells that received no toxin were considered as 100 % and used to calculate the percent viability of other data points. Insets show western blots for *in vitro* ADP-ribosylation assay with the cell lysates. In the inset, top panels show ADP-ribosylated eEF-2 while the bottom panel shows the blot using antibody against the carboxy terminus of eEF-2 of human origin. − and + denote the absence or addition of FP59 respectively.

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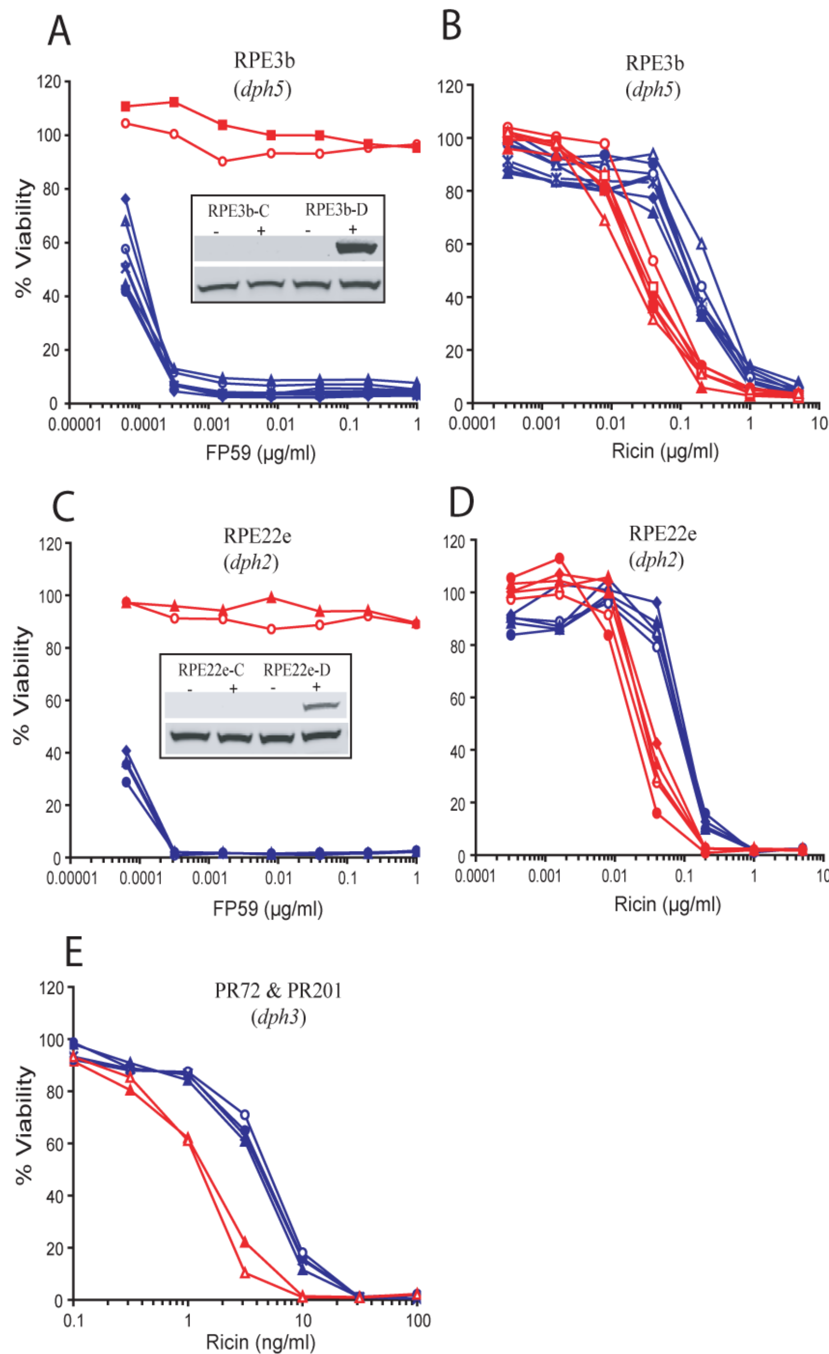

Fig. 3. Toxin sensitivity of diphthamide-deficient CHO mutant cells complemented by transfection of the corresponding *DPH* genes

Cells transfected with corresponding *DPH* gene have been designated by adding "D" after the name of cell lines and are shown in blue, while control transfectants for same cell types have been shown by adding "C" and are shown in red. Each line is derived from an independent stable transformant cell line. Toxin sensitivities of representative individual clones were measured as in Fig. 2 for "PA + FP59" (a, c) and "ricin" (b, d, e). In panels b, d, and e the blue lines ("D" clones) are clustered to the right, indicating increased resistance to ricin. Insets show the western blot for *in vitro* ADP-ribosylation assays done with the cell lysates, as in Fig. 2. In the experiment of panel e, the "-C" clones are those transfected with an unrelated gene,

VPS11, rather than empty vector. Also, in the experiment of panel e, both mutant and transfected cells show increased sensitivity towards ricin compared to other experiments (e.g., Fig. 2, 3b and 3d) due to use of a more potent batch of ricin, but the effect of complementation was again to increase resistance.

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Table 1

Ricin sensitivity of diphthamide-deficient CHO cells and their parental cells.

CHO Cell Line	Gene mutated	Parental cells	#EC50 (ng/ml)	*Ratio	Reference
WTP4	-	-	115	-	Liu and Leppla, 2003
PR72	*DPH3*	WTP4	38	3	Liu and Leppla, 2003
PR201	*DPH3*	WTP4	39	3	Liu and Leppla, 2003
CHO-K1	-	-	120	-	Moehring et al., 1984
RPE3b	*DPH5*	CHO-K1	40	3	Moehring et al., 1980
RPE22e	*DPH2*	CHO-K1	40	3	Moehring et al., 1980

All the cell lines used are derivatives of CHO cells and were obtained by chemical or retroviral mutagenesis as reported earlier (Liu and Leppla, 2003).

EC_{50} is the effective concentration of toxin required to kill 50% of cells. Data is taken from Fig. 2.

* Ratio was calculated by dividing EC_{50} value of parental cells by the EC_{50} of their corresponding diphthamide-deficient cells.

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Table 2

Ricin sensitivity of diphthamide-deficient cells transfected with the corresponding *DPH* gene or control vector.

CHO Cell Line	Transfected with	#EC_{50} of individual transfectants [ng/ml] (average EC_{50})	*Ratio	*p* Value
RPE3b-D	*DPH5* gene	130,100,120,100,300,120,150,120 (143)	4.7	0.0014
RPE3b-C	Empty vector	25,25,32,20,30,50 (30)		
RPE22e-D	*DPH2* gene	100,90,80,90, 90 (90)	3.3	<.0001
RPE22e-C	Empty vector	22,28,20,35,30 (27)		
PR72-D, PR201-D& (2 clones each)	*DPH3* gene	4.5, 4, 5, 4.5 (4.5)	3.2	0.0001
PR72-C, PR201-C&	Unrelated gene, *VPS11*	1.5, 1.2 (1.4)		

Cell lines were generated by transfecting the diphthamide-deficient CHO cells either with the corresponding *DPH* gene (cell line names having the extension "-D") or with the control (empty vector or unrelated *VPS11* gene; cell line names having the extension "-C").

#EC_{50} is the effective concentration of toxin required to kill 50 % of cells. Data is taken from Fig. 3.

*Fold difference was calculated by dividing average EC_{50} of *DPH* transfectants by the average of control transfectants.

&Assays for these cell lines used a more potent batch of ricin than that used for the other toxicity assays.