

Mass Spectral Detection of Diethoxyphospho-Tyrosine Adducts on Proteins from HEK293 Cells Using Monoclonal Antibody depY for Enrichment

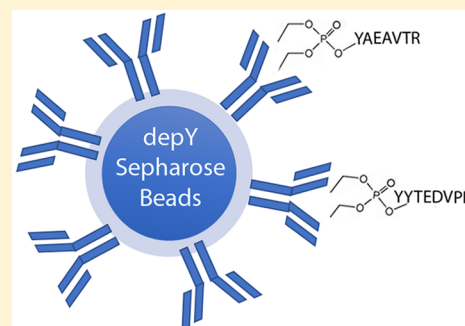
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ABSTRACT: Chronic illness from exposure to organophosphorus toxicants is hypothesized to involve modification of unknown proteins. Tyrosine in proteins that have no active site serine readily reacts with organophosphorus toxicants. We developed a monoclonal antibody, depY, that specifically recognizes diethoxyphospho-tyrosine in proteins and peptides, independent of the surrounding amino acid sequence. Our goal in the current study was to identify diethoxyphosphorylated proteins in human HEK293 cell lysate treated with chlorpyrifos oxon. Cell lysates treated with chlorpyrifos oxon were recognized by depY antibody in ELISA and capillary electrophoresis based Western blot. Tryptic peptides were analyzed by liquid chromatography tandem mass spectrometry. Liquid chromatography tandem mass spectrometry identified 116 diethoxyphospho-tyrosine peptides from 73 proteins in immunopurified samples, but found only 15 diethoxyphospho-tyrosine peptides from 12 proteins when the same sample was not immunopurified on depY. The most abundant proteins in the cell lysate, histone H4, heat shock 70 kDa protein 1A/1B, heat shock protein HSP 90 β , and α -enolase, were represented by several diethoxyphospho-tyrosine peptides. It was concluded that use of immobilized depY improved the number of diethoxyphospho-tyrosine peptides identified in a complex mixture. The mass spectrometry results confirmed the specificity of depY for diethoxyphospho-tyrosine peptides independent of the context of the modified tyrosine, which means depY could be used to analyze modified proteins in any species. Use of the depY antibody could lead to an understanding of chronic illness from organophosphorus pesticide exposure.



INTRODUCTION

Historically, the cholinesterases have been recognized as the principal targets for irreversible inhibition by organophosphate toxicants.¹ By the late 1950s, the residue labeled by diisopropylfluorophosphate was determined to be serine for both acetylcholinesterase² (AChE) and butyrylcholinesterase³ (BChE). In 1963, Sanger reported amino acid sequences in the vicinity of the labeled serine for both enzymes.⁴ These sequences were confirmed in the mid-1980s for AChE⁵ and for BChE.^{6,7}

Despite a prodigious literature devoted toward elucidating interactions of organophosphorus toxicants with the active-site serine of the cholinesterases, it was recognized early on that cholinesterase inhibitors produce a variety of pharmacological effects which cannot be attributed solely to the inhibition of cholinesterase. In their review from 1949, Koelle and Gilman stated “The fact that a compound is capable of inhibiting cholinesterase does not imply that it produces its pharmacological effects by this mechanism” alone.¹ This concept was resurrected in the late 1990s^{8–11} and serves as a cornerstone for a line of investigation that we have been pursuing for the past

12 years, namely tyrosine and lysine targets for organophosphorus toxicants.

In 1963 Sanger demonstrated that diisopropylfluorophosphate could label tyrosine in human serum albumin, by sequencing the labeled peptide: ArgTyrThrLys.⁴ This observation was confirmed in 2005.¹² Shortly after Sanger’s report, other reports on the reaction of organophosphates with tyrosine on proteins appeared.^{13–15} More recently we have identified organophosphate-modified tyrosine and lysine on peptides from 13 proteins, suggesting that proteins with no enzyme activity and no active site serine are targets of organophosphorus toxicants.^{16,17}

Not all tyrosines in a given protein react with organophosphates. Reactivity of tyrosine appears to be facilitated by the presence of a positively charged amino acid within 6 Å of the tyrosine.^{16,18} Once formed, the organophospho-tyrosine adduct is stable. Diethoxyphospho-tyrosine on human albumin lost only 25% of the label after 8 months at pH 7.4 and 22 °C.¹⁹

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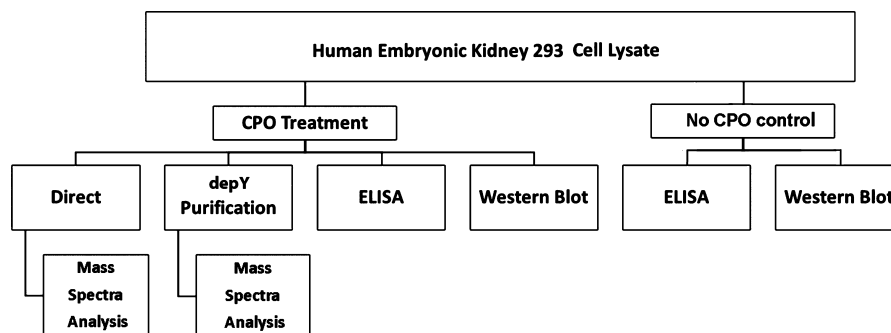


Figure 1. Workflow scheme. Cell lysate with a protein concentration of 4.9 mg/mL was treated with chlorpyrifos oxon (CPO). A portion of the CPO-treated cell lysate was digested with trypsin and analyzed by LC-MS/MS with or without prior immunopurification of tryptic peptides on immobilized monoclonal antibody depY. Intact CPO-treated cell lysate (no trypsin) and control cell lysate (no CPO, no trypsin) were analyzed for reactivity with monoclonal depY by ELISA and capillary electrophoresis-Western blot.

Diethoxyphospho-tyrosine and phosphonylated-tyrosine adducts produced by reaction with soman, sarin, cyclosarin, tabun, and VX do not undergo dealkylation as does organophospho-serine on cholinesterases, a process called aging.^{17,20,21} An exception is the dimethoxyphospho-tyrosine adduct (dichlorvos product) which does age to the monomethoxyphospho-tyrosine adduct.¹⁸ These features make organophospho-tyrosines attractive candidates for identifying novel proteins that react with organophosphates, and reasonable candidates for production of antibodies.

To facilitate our search for proteins that are organophosphorylated on tyrosine, we produced a monoclonal antibody, depY, that selectively recognizes diethoxyphospho-tyrosine.²² The depY antibody recognizes diethoxyphospho-tyrosine independent of the amino acid sequence around the modified tyrosine. It does not recognize diethoxyphospho-lysine, diethoxyphospho-serine, phospho-serine, phospho-threonine, phospho-tyrosine, dimethoxyphospho-tyrosine (dichlorvos adduct), monomethoxyphospho-tyrosine (aged dichlorvos adduct), dimethoxyphospho-serine, or cresylphosphoserine.

The goal of the current study was to identify diethoxyphosphorylated peptides from a HEK293 cell lysate that was treated with chlorpyrifos oxon (CPO). We compared results for samples that were immunopurified to samples that were not immunopurified before analysis by mass spectrometry. Immunopurification of tryptic peptides with depY decreased the total number of peptides identified by 50- to 70-fold, increased the number of diethoxyphospho-tyrosine peptides detected by 8-fold, and excluded peptides diethoxyphosphorylated on lysine or serine. We identified 116 different diethoxyphospho-tyrosine peptides from 73 different proteins.

MATERIALS

Mouse monoclonal depY against diethoxyphospho-tyrosine was produced in house.²² The heavy and light chain sequences of depY are deposited in the NCBI database with Genbank accession numbers MG182361 and MG182362. The following were from Thermo Fisher Scientific: dithiothreitol (Electrophoresis grade, no. 172) stored at -20°C ; urea (for molecular biology, 327380010); formic acid (Optima LC/MS A117); acetonitrile (DNA sequencing, BP1170). The following were from Sigma-Aldrich: bovine serum albumin Fraction V, A-8022, stored at 4°C ; iodoacetamide I-6125, stored at 4°C ; trypsin (porcine sequencing grade modified, Promega no. V511C) stored at -80°C . CNBr-activated Sepharose 4 Fast Flow was from Amersham Bioscience GE Healthcare Life Sciences 17-0981, stored at 4°C . Antimouse IgG conjugated to horseradish peroxidase was from

Cell Signaling 7076, stored at -20°C . 3,3',5,5'-Tetramethylbenzidine was from BioLegend 421501. Trifluoroacetic acid sequencing grade was from Beckman 290204. Chlorpyrifos oxon 98% pure was from Chem Service Inc. MET-11459B, stored at -80°C . Additional materials are described below in sections where the materials were used.

METHODS

Cell Culture. Human HEK 293 cells (ATCC CRL-1573) were cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific 11965-092) at 37°C in a humidified atmosphere with 5% CO_2 .

Cell Lysates and Chlorpyrifos Oxon Treatment. Cells were grown in T75 flasks until they were confluent. Cell lysates were generated using RIPA buffer (25 mM TrisCl pH 7.6, 150 mM sodium chloride, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Thermo Scientific, cat. no. 89901). Cells from three T75 flasks were mixed with 2.1 mL RIPA buffer, sonicated at 4°C with a Sonic Dismembrator Model 500 (Fisher Scientific) at 20% power for 10 s, and allowed to cool for 10 s. The cycle was repeated three times. Soluble fractions were collected by centrifugation for 20 min at 14,000g. Protein concentrations of cell lysates were determined by using the BCA Protein Assay Kit (Thermo Scientific, cat. no. 23225). Cell lysate (4.9 mg protein per mL) was divided into five 0.4 mL lots. One lot was used for unlabeled control. The other four lots were incubated with 10, 100, 250, or 1000 μM chlorpyrifos oxon (CPO) at room temperature for 24 h. Freshly prepared CPO solutions in ethanol had concentrations of 300, 100, 30, and 3 mM. A 1.4 μL aliquot of 300 mM CPO added to 0.4 mL cell lysate yielded 1000 μM CPO. A 1 μL aliquot of 100 mM CPO and a 1.4 μL aliquot of 30 and 3 mM CPO added to 0.4 mL cell lysate yielded CPO concentrations of 250, 100, and 10 μM . Untreated cell lysate and a portion of each treated cell lysate were used for ELISA and Western Blot. The remainder from each CPO treated cell lysate was digested with trypsin and further divided into two lots for mass spectrometry. The tryptic peptides in one lot were immunopurified with depY antibody in preparation for LC-MS/MS, while peptides in the other lot were not immunopurified, but were used directly for LC-MS/MS as an unextracted control. Figure 1 diagrams how the 4.9 mg/mL cell lysate was divided for analysis by LC-MS/MS, ELISA, and Western blotting.

ELISA. Immulon 96-well plates (2HB flat bottom, Thermo Fisher Scientific, Milford MA, cat. no. 3455) were coated with 1 μg cell lysate per well in 100 μL of pH 9.6 coating buffer (3 g sodium carbonate and 6 g sodium bicarbonate in 1 L water) at 4°C overnight. Wells were blocked with 1% bovine serum albumin (BSA) in Tris buffered saline (TBS: 20 mM TrisCl, 0.15 M sodium chloride pH 7.4) at room temperature for 1 h, followed by one wash with TBS containing 0.05% Tween-20 (TBST). Monoclonal depY diluted to 0.02 $\mu\text{g}/100 \mu\text{L}$ in 1% BSA/TBS was added to the wells. The plate was rocked for 2 h at room temperature, washed 3 times with TBST, and incubated with horseradish peroxidase (HRP) conjugated antimouse IgG (5 μL

diluted into 20 mL of 1% BSA/TBS) for 2 h at room temperature. After the plate was washed 5 times with TBST, enzyme activity of horseradish peroxidase was developed with 100 μ L of 3,3',5,5'-tetramethylbenzidine per well. The HRP reaction was stopped after 20 min by addition of 100 μ L of 0.16 M sulfuric acid. The intensity of the yellow product was measured at 405 nm on a BioTek 96-well plate reader (Winooski, VT).

Capillary Electrophoresis-Western Blot. The Simple Western Testing Service personnel at RayBiotech, Inc. (Norcross, GA, USA) performed Western blot analysis on HEK293 cell lysates using a WES capillary electrophoresis device (ProteinSimple, San Jose, CA, USA). HEK293 cell lysates with a protein concentration of 4.9 mg/mL had been treated with 0, 10, 100, 250, or 1000 μ M CPO. The cell lysates were diluted to 0.2 mg/mL by RayBiotech personnel, who injected 40 nL of the diluted samples into the capillary electrophoresis device. After electrophoresis, proteins were immobilized to the capillary wall via a proprietary, photoactivated capture chemistry. Matrix was removed, and the depY antibody at a concentration of 0.02 mg/mL was passed through the capillary. Proteins that bound the depY antibody were located using an HRP-conjugated antimouse IgG secondary antibody (ProteinSimple 042-205) together with a chemiluminescent substrate (ProteinSimple PS-CS01).

Monoclonal depY Immobilized on Sepharose Beads. CNBr-activated Sepharose (1 g) washed and swollen to 3 mL was coupled to 4.7 mg of depY antibody in 1 mL of 0.15 M sodium bicarbonate, 0.5 M sodium chloride pH 8. It was estimated that 4.5 mg of the antibody was covalently bound based on absorbance at 280 nm of the antibody solution before and after the binding reaction. The beads were washed with pH 8 buffer, 1 M sodium chloride, pH 3.5 buffer, and phosphate buffered saline (PBS). Beads were stored in 15 mL of PBS, 0.1% azide, at 4 °C. A 0.1 mL suspension contained 20 μ L beads covalently bound to 30 μ g of depY.

Sample Preparation for Mass Spectrometry. The sample preparation method was adapted from Wisniewski et al.²³ Disulfide bonds in cell lysates (380 μ g protein, control, and CPO-treated) were reduced by addition of 10 mM dithiothreitol in 0.1 M TrisCl pH 8.5 plus 8 M urea (final volume 100 μ L). These solutions were vortexed and heated in a boiling water bath for 3 min. Each sample was mixed with 200 μ L of 8 M urea in 0.1 M TrisCl pH 8.5, placed in a Microcon-YM10 centrifugal filter (Ultracel 10 kDa MWCO, Millipore Sigma, Tullagreen Ireland, cat. no. MRCPR010), and centrifuged at 14,000g for 10 min until the sample compartment was nearly dry. Excess dithiothreitol was washed off with 400 μ L of 8 M urea, centrifuged at 14,000g for 15 min until the filter was nearly dry. This process was repeated twice. Sulfhydryl groups were carbamidomethylated by adding 100 μ L of 55 mM iodoacetamide in 0.1 M TrisCl pH 8.5 plus 8 M urea to protein on the filter, vortexed for 1 min, and incubated in the dark for 20 min. Excess iodoacetamide was washed out of the filter units by centrifugation at 14,000g for 10 min, followed by two washes with 100 μ L of 8 M urea. The contents of each filter unit were equilibrated with 100 μ L of 50 mM ammonium bicarbonate pH 8 and centrifuged at 14,000g for 10 min. This step was repeated twice. The contents of each filter were suspended in 100 μ L of 50 mM ammonium bicarbonate and mixed with 10 μ L of 0.4 mg/mL trypsin (trypsin to protein ratio about ~1:100 μ g/ μ g). Tryptic digestion was performed on the filter, in a humidified chamber, at 37 °C, overnight (16–18 h). Filter units were centrifuged at 14,000g for 10 min into new tubes to collect the tryptic peptides, rinsed with 100 μ L of 0.5 M sodium chloride, and centrifuged at 14,000g for 10 min collecting the flow through into the same tubes. Peptides were dried using a SpeedVac vacuum centrifuge and resuspended with 1 mL of 0.4% formic acid pH 3. Acidified peptides were desalted on MCX cartridges (OASIS MCX CC-30 mg, Waters, Taunton MA, cat. no. 186000252) that had been equilibrated with a 1:1 methanol:water solution. Cartridges were washed with 1 mL of 5% methanol, 0.1% formic acid, followed by 1 mL of 100% methanol. Peptides were eluted from MCX cartridges with 1 mL of fresh elution buffer (50 μ L of 28% ammonium hydroxide plus 950 μ L of 100% methanol). The 1 mL eluates containing the tryptic peptides were divided equally into two tubes and dried. The contents of one tube were dissolved in 20 μ L of 0.1%

formic acid (unenriched samples, about 10 μ g protein per μ L assuming no losses) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The contents of the other tubes were enriched for diethoxyphospho-tyrosine peptides by using depY antibody attached to Sepharose, as follows: Tryptic peptides from diethoxyphospho-tyrosine-labeled cell lysates were incubated with 40 μ L of depY Sepharose beads in PBS for 2 h. Beads were placed in a 0.45 μ m spin filter (Ultra free MC HVLP, Millipore-Sigma, cat. no. UFC30HV00) and washed 3 times with 0.5 mL PBS, followed by 3 washes with 0.5 mL water. Bound peptides were released with 50% acetonitrile/1% trifluoroacetic acid, dried in a SpeedVac, and redissolved in 20 μ L of 0.1% formic acid for LC-MS/MS analysis (enriched sample, <10 μ g protein per μ L).

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) on the Triple-TOF 6600. Mass spectral data were acquired on two different mass spectrometers: a Triple-TOF 6600 (ABI Sciex, Framingham, MA) and an Orbitrap Fusion Lumos (Thermo Scientific, Rockford, IL).

Peptides were introduced into the Triple-TOF 6600 mass spectrometer using ultrahigh-pressure liquid chromatography. Fifty μ g or less of sample, in a 5 μ L volume, was loaded. A splitless Ultra 1D Plus ultrahigh-pressure chromatography system (Eksigent, Dublin, CA) was coupled to the Triple-TOF via a cHiPLC Nanoflex microchip column system (Eksigent, Dublin, CA). The Nanoflex system used a replaceable microfluidic trap column and a replaceable separation column. Both were packed with ChromXP C₁₈ (3 μ m, 120 Å particles; Trap: 200 μ m \times 0.5 mm; Separation: 75 μ m \times 15 cm). Chromatography solvents were water/acetonitrile/formic acid (A: 100/0/0.1%, B: 0/100/0.1%). Trapping and desalting were carried out at 2 μ L/min for 15 min with 100% mobile phase A. Separation was obtained with a linear gradient 5%A/95%B to 70%A/30%B over 60 min at a flow rate of 0.3 μ L/min.

The Triple-TOF 6600 mass spectrometer was fitted with a Nanospray III source (AB SCIEX, Framingham, MA) and a Pico Tip emitter (no. FS360-20-10-N-5-C12, New Objectives, Woburn, MA). Mass spectra were collected in positive mode, over a mass range from 200 to 2000 m/z , using an accumulation time of 250 ms, a collision energy of 10 V, a declustering potential of 60 V, an ion spray potential of 2700 V, and an interface heater temperature of 150 °C. Peptide fragmentation was accomplished by collision-induced dissociation using nitrogen as the collision gas at a pressure of 2×10^{-5} Torr. Fragmentation spectra were collected in positive mode, over a mass range of 50–2000 Da, using an accumulation time of 25 ms, a collision energy determined by the software (rolling), and a collision energy spread of ± 15 V. Peptides to be fragmented were chosen by an information directed acquisition algorithm using charge state 1–4 and minimum signal of 100 cps. Up to 50 fragmentation spectra were collected in each cycle with target ions being excluded for 5 s after the second acquisition. Masses within 6 Da of a target ion were excluded.

Database searching of the Triple TOF data used the Paragon algorithm 5.5²⁴ in Protein Pilot software 4.0.8085 (AB Sciex, Framingham, MA). Database search parameters included sample type = identification, Cys alkylation = iodoacetamide, digestion = trypsin, instrument = triple TOF 6600, special factors = organophosphorylation, species = *Homo sapiens*, ID focus = biological modifications, database = uniprot_sprotJAN2015.fasta, search effort = thorough, and FDR analysis = active. A new section entitled organophosphorylation was added to the Special Factors portion of the Paragon algorithm. This change required modification of the Parameter Translation.xml and Protein Pilot Data Dictionary.xml files to include searches for diethoxyphospho-adducts (+136.03 amu) on tyrosine, lysine, serine, threonine, histidine, and cysteine as well as monoethoxyphospho-adducts (+108.00 amu) on tyrosine and serine.

Sequences of peptides and the locations of the labeled residues that were identified by Protein Pilot were confirmed by manual sequencing using PeakView v 2.1 (AB Sciex, Framingham, MA).

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) on the Orbitrap Fusion Lumos. Peptides were introduced directly into the Orbitrap mass spectrometer using an Ultimate 3000

UHPLC (Dionex, Sunnyvale, CA). Chromatography employed an Acclaim PepMap RSLC C18 column (75 $\mu\text{m} \times 150\text{ mm}$) (Thermo Scientific no. 164534) and an Acclaim PepMap 100 trap column (75 $\mu\text{m} \times 20\text{ mm}$) (Thermo Scientific no. 164535). One μg of the enriched digests or 2 μg of the unenriched digests was loaded onto the trap column. Chromatography solvents were water/acetonitrile/formic acid (A: 100/0/0.1%, B: 0/100/0.1%). Trapping and desalting were carried out at 4 $\mu\text{L}/\text{min}$ with 100% solvent A. Separation was obtained with a linear gradient 3%A/97%B to 55%A/45% B over 90 min at a flow rate of 0.3 $\mu\text{L}/\text{min}$.

The OrbiTrap Fusion Lumos was fitted with a Nanospray Flex NG ion source (Thermo Scientific) and a stainless steel Nanobore emitter (Thermo Finnigan #ESS42). Mass spectra were collected in positive mode, over a mass range from 400 to 1600 m/z , at a resolution of 120,000. Parent ions were resolved in the OrbiTrap. The top 10 peptides were selected for fragmentation. The dynamic exclusion repeat count was one with a repeat duration of 20 s. Fragmentation was performed by both collision-induced dissociation (CID) and high-energy collision-induced dissociation (HCD). CID was performed in the ion trap using a minimum threshold of 50,000; isolation width of 2 m/z , normalized energy of 35 V; activation Q of 0.25, default charge state of 2, and an activation time of 10 ms. HCD was performed in the OrbiTrap using a minimum threshold of 50,000, resolution of 60,000, isolation width of 2 m/z , normalized energy of 44 V, default charge state of 2, and an activation time of 0.1 ms.

Database searching of the OrbiTrap data used Sequest HT algorithm in Proteome Discoverer 2.1. The processing workflow included database = *Homo sapiens* SwissProt v2017-06-07; protease = trypsin (full); max missed cleavage sites = 2; peptide length = 6 to 144; precursor mass tolerance = 10 ppm; fragment mass tolerance = 0.6 Da; variable modifications = oxidized methionine, phospho-serine, phospho-threonine, phospho-tyrosine, diethylphospho-serine, diethylphospho-threonine, diethylphospho-tyrosine, diethylphospho-lysine, diethylphospho-cysteine, diethylphospho-histidine, ethylphospho-serine, ethylphospho-threonine, ethylphospho-tyrosine, ethylphospho-lysine, and ethylphospho-cysteine, fixed modification = carbamidomethylated cysteine, and percolator validator with decoy sequences from Waters Protein Lynx Global server 2.5.

RESULTS

ELISA. The first goal was to determine whether monoclonal antibody depY recognizes CPO-labeled proteins in CPO-treated HEK 293 cell lysates. Figure 2 shows that the ELISA signal increased progressively as the concentration of CPO used to label the cell lysate increased from 100 to 1000 μM . The

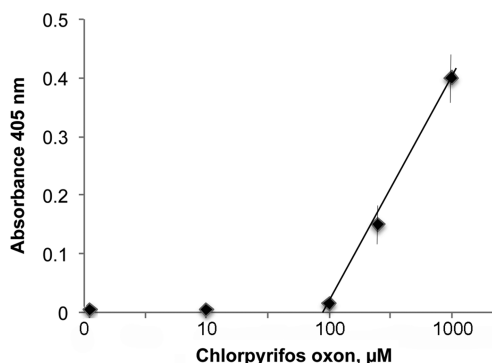


Figure 2. ELISA analysis of the interaction of monoclonal antibody depY with CPO-labeled proteins in HEK293 cell lysates treated with 0, 10, 100, 250, or 1000 μM CPO. The depY antibody concentration was 0.02 μg in 100 μL of 1% BSA/TBS. Data points are the mean of four measurements \pm SD. Data were corrected for blank signals ($A_{405\text{ nm}} = 0.069$). The negative control cell lysate not treated with CPO, indicated as 0 μM CPO, had the same signal intensity as background.

semilog plot in Figure 2 goes through zero at 95 μM CPO. This is interpreted to mean that 95 μM CPO is consumed by something other than tyrosine. The possible CPO consumers include residues other than tyrosine, the RIPA buffer, and the plastic microfuge tube. The signal from the negative control cell lysate, not treated with CPO, was the same as background, indicating the absence of nonspecific antibody binding. It was concluded that monoclonal antibody depY binds specifically to CPO-labeled proteins in HEK293 cell lysates.

Capillary Electrophoresis-Western Blot. Proteins in the HEK293 cell lysates treated with 0, 10, 100, 250, and 1000 μM CPO were separated by capillary electrophoresis and immobilized on the capillary wall. Reaction of the proteins with depY is shown in Figure 3A. Samples treated with 0 and 10 μM CPO had a weak signal at a molecular weight of 234 kDa representing background. The 100 μM CPO-treated sample had a weak signal at 56 kDa. The 250 μM CPO-treated sample had weak signals at 55, 71, 95, 151, and 228 kDa. The 1000 μM CPO-treated sample had strong signals at 36, 45, 56, 146, 172, and 202 kDa. Figure 3B is a blot representation of the data in Figure 3A. It was concluded that capillary electrophoresis-Western blotting confirmed the ELISA results, namely that monoclonal antibody depY recognizes CPO-labeled proteins in CPO-treated HEK 293 cell lysate. The Western blot indicated that many proteins with a range of molecular weights were diethoxyphosphorylated on tyrosine. This preliminary conclusion was confirmed by mass spectrometry results presented in the next sections.

LC-MS/MS Mass Spectrometry. ELISA and Western blotting results provided evidence that proteins had been modified by CPO on tyrosine. Our previous work had demonstrated that depY specifically recognizes diethoxyphospho-tyrosine independent of the neighboring amino acid sequence and that depY does not recognize diethoxyphospho-lysine or other alternatives.²² Our next goal was to identify the CPO-modified proteins. To achieve this goal, we digested the proteins with trypsin and analyzed the peptides by LC-MS/MS. Digests were run on two different mass spectrometers.

When the tryptic digest of the 1000 μM CPO-treated HEK293 cell lysate was immunopurified with depY a total of 116 different diethoxyphospho-tyrosine containing peptides from 73 different proteins were identified (Table 1). This list was restricted to peptides that were assigned a confidence score of 90% or greater by Protein Pilot or a Posterior Error Probability (PEP) score ≤ 0.01 by Sequest HT. Forty peptides, from 23 proteins, were detected by the Triple TOF/Protein Pilot. One hundred peptides, from 68 proteins, were detected by the OrbiTrap/Proteome Discoverer. Twenty-four peptides were detected by both instruments resulting in 116 unique peptides. Four times more diethoxyphosphorylated peptides were detected by the OrbiTrap than by the Triple TOF mass spectrometer. The sample for the OrbiTrap contained 1 μg protein digest in 5 μL , whereas the sample for the Triple TOF mass spectrometer contained 50 μg protein digest in 5 μL . MS/MS spectra for selected peptides were manually sequenced to confirm the assignments.

When this sample was not immunopurified with depY, only seven diethoxyphospho-tyrosine containing peptides (from six different proteins) were identified by the Triple TOF, and eight diethoxyphospho-tyrosine containing peptides (from six different proteins) were identified by the OrbiTrap. One peptide containing diethoxyphosphorylated-lysine was detected by the Triple TOF and two by the OrbiTrap. These differences reflect

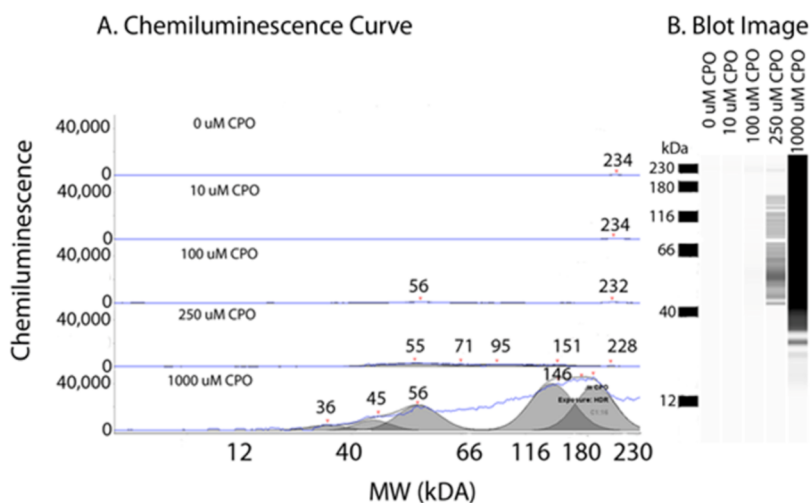


Figure 3. Capillary electrophoresis-based Western blot analysis (RayBiotech) of the interaction of monoclonal antibody, depY, with diethoxyphospho-tyrosine-labeled proteins from HEK293 cell lysates. Samples containing 0.008 μg protein in 40 nL were hybridized with 0.02 mg/mL monoclonal antibody depY. (A) An electropherogram of the depY-captured proteins. (B) A blot representation of the same samples including a molecular weight standard lane.

the specificity of depY for diethoxyphospho-tyrosine and confirm our previous report that depY does not recognize diethoxyphospho-lysine and diethoxyphospho-serine. Furthermore, they demonstrate the ability of depY to improve the number of diethoxyphospho-tyrosine peptides identified in a complex mixture. Eight-fold-more diethoxyphospho-tyrosine containing peptides were found when the samples were enriched by extraction with depY.

Use of depY decreased the complexity of the peptide mixture. For example, when the tryptic digest of the HEK293 cell lysate (treated with 1000 μM CPO) was submitted for mass spectral analysis on the Triple TOF mass spectrometer without depY treatment, a total of 44,921 peptides were detected with confidence values >90%. This number includes peptides that appeared more than once in the data set. Twenty-nine of these peptides were diethoxyphosphorylated. In contrast, when the tryptic digest was immunopurified with depY a total of 629 peptides were detected with confidence >90%, 109 of which were diethoxyphospho-tyrosine peptides. Higher numbers for diethoxyphospho-tyrosine peptides are reported here than in the above analysis of unique diethoxyphospho-tyrosine peptides because duplicate peptides are included here. Without depY extraction 0.06% of the total peptides were diethoxyphosphorylated [$29 \div 44,921 \times 100$]. With depY extraction, 17% of the peptides were diethoxyphosphorylated [$109 \div 629 \times 100$], a 280-fold enhancement [$17 \div 0.06$]. The total number of peptides in the depY extracted sample was 70-fold lower than in the unextracted sample [$44,921 \div 629$]. Similar results were obtained for the samples treated with 250 and 10 μM CPO.

The number of times that the mass spectrum for each labeled peptide was recorded, that is, the peptide count, is a measure of the relative amount of each labeled peptide present in the digest. In the Triple TOF data, as the concentration of CPO used in the labeling decreased, both the peptide count for a given peptide decreased (in general), and the number of labeled peptides decreased. Only two diethoxyphospho-tyrosine peptides were detected in the 10 μM CPO-treated cell lysate; the peptides were from histone H4 (P62805) and histone H1.4 (P10412).

The relative abundance of each labeled protein within the HEK293 proteome is indicated in Table 1 by its rank. The rank is based on the iBAQ number (intensity-based absolute quantification) for each protein.²⁷ iBAQ numbers were assigned to 6858 HEK293 proteins in the study by Geiger et al.²⁵ in their supplemental Table S4. The most abundant protein has rank 1, and the least abundant has rank 6858. Table 1 shows that the majority of the proteins identified in our study ranked between 1 and 200 (within the top 3%), only 7 rank above 1000 (below 14%). This indicates that we identified primarily the more abundant proteins. The rank of each protein in terms of abundance within the HEK293 proteome is given in Table 1.

The sequences for all the peptides from the Triple TOF portion of the study were confirmed by manual sequencing, and the locations of the labeled residues determined. An example is the fragmentation spectrum for diethoxyphosphorylated peptide YYPTEDVPR from ribosomal protein L6 shown in Figure 4. The doubly charged parent ion has a mass of 638.2 Da consistent with an added mass of 136 from diethoxyphosphate. Two tyrosines are in the peptide. Proof that the adduct is on the second tyrosine is the 1112.5 Da mass of the y_8 ion. If the adduct had been on the N-terminal tyrosine, the y_8 ion would have had a mass of 976.4 Da. These calculations were made with the aid of the Proteomics Toolkit (<http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html>).

DISCUSSION

Selectivity of the depY Antibody. The ELISA, Western blot, and mass spectral analyses all indicated that monoclonal antibody depY selectively reacts with diethoxyphospho-tyrosine proteins and peptides in HEK293 cell lysates. The Western blot indicated that multiple CPO-labeled proteins reacted with depY, consistent with the mass spectral results. CPO can diethoxyphosphorylate tyrosine²⁸ and lysine²⁹ in addition to serine residues. Diethoxyphospho-lysine was detected in the lysate that was not immunopurified with depY. Mass spectral analysis of CPO-labeled peptides that were immunopurified with depY yielded diethoxyphospho-tyrosine adducts, but not diethoxyphosphorylated lysine or serine. This result is

Table 1. Immunopurified Peptides from HEK293 Cell Lysate Treated with 1 mM CPO^a

| protein name | access no. | peptide sequence ^b | residue | rank ^c | PhosphoSite ^d |
|---|------------|--|---------|-------------------|--------------------------|
| actin, cytoplasmic 2 | P63261 | DSYVGD ^Y DEAQS ^K | Y53 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | DLTDY ^L LMK | Y188 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | GYSFTT ^T TAER | Y198 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | QF ^Y YDESGPSIVHR | Y362 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | SYELPDGQVITIGNER | Y240 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | DLYANTVLSGGTTMYPGIADR | Y294 | 1710 | yes |
| actin, cytoplasmic 2 | P63261 | IWHHTFY ^N NELR | Y91 | 1710 | yes |
| actin, α cardiac muscle 1 | P68032 | YPIEHGIITNWDDMoxEK | Y71 | 156 | yes |
| ADP/ATP translocase 2 | P05141 | YFPTQALNFAFK | Y81 | 101 | yes |
| poly [ADP-ribose] polymerase 1 | P09874 | FYTLIPHDFGMoxK | Y737 | 151 | yes |
| adenine phosphoribosyl transferase | P07741-1 | IDYIAGLDSR | Y60 | 392 | yes |
| α -enolase | P06733 | AAVPSGASTGIYEAL ^E LR | Y44 | 14 | yes |
| α -enolase | P06733 | IGAEVY ^H NL ^K | Y189 | 14 | yes |
| α -enolase | P06733 | AGYTDKVVIGMDVAASEFFR | Y236 | 14 | yes |
| α -enolase | P06733 | YISPDQLADLYK | Y270 | 14 | yes |
| α -enolase | P06733 | DYPVVSIEDPFDQDDWGAWQK | Y287 | - | yes |
| ATP synthase subunit α , mitochondrial | P25705-1 | EAYPGDVFY ^L HSR | Y337 | 225 | yes |
| ATP synthase subunit α , mitochondrial | P25705-1 | EAYPGDVFY ^L HSR | Y343 | 225 | yes |
| ATP synthase subunit β , mitochondrial | P06576 | IMDPNIVGSEHY ^D VAR | Y418 | 141 | yes |
| ATP synthase subunit γ , mitochondrial | P36542-1 | IYGLGSLALY ^E K | Y69 | 637 | no |
| calpain small subunit 1 | P04632 | YSDESGNMoxDFDNFISCamLVR | Y217 | 1390 | no |
| cofilin-1 | P23528 | EILVGDVGTVD ^D DPYATFVK | Y68 | 25 | yes |
| cofilin-1 | P23528 | HELQANCY ^E EEVK | Y140 | 25 | yes |
| cofilin-1 | P23528 | YALYDATY ^E ETK | Y82 | 25 | yes |
| cofilin-1 | P23528 | YALYDATY ^E ETK | Y85 | 25 | yes |
| cofilin-1 | P23528 | YALYDATY ^E ETK | Y89 | 25 | yes |
| elongation factor 1- α 1 | P68104 | YYVTIIDAPGHR | Y85 | 8 | yes |
| elongation factor 1- α 1 | P68104 | YYVTIIDAPGHR | Y86 | 8 | yes |
| elongation factor 1- α 1 | P68104 | EHALLAY ^T LGVK | Y141 | 8 | yes |
| elongation factor 1- α 1 | P68104 | IGY ^N PD ^T VAFVPISGWNGDNMoxLEPSANMoxPWFK | Y183 | 8 | no |
| elongation factor 2 | P13639 | YFDPANGK | Y265 | 26 | yes |
| Isoform 2 of elongation factor 1- γ | P26641-2 | ILGLLDAY ^L LK | Y145 | 100 | yes |
| putative elongation factor 1- α -like 3 | Q5VTE0 | CVESFSDY ^P PLGR | Y418 | 8 | nd |
| endoplasmic | P14625 | DISTNY ^Y ASQK | Y678 | 228 | yes |
| glyceraldehyde-3-phosphate dehydrogenase | P04406-1 | LISWY ^D NEFGYSNR | Y314 | 12 | yes |
| glyceraldehyde-3-phosphate dehydrogenase | P04406-1 | LISWY ^D NEFGY ^S NR | Y320 | 12 | yes |
| GTP-binding nuclear protein RAN | P62826 | NLQY ^Y DISAK | Y146 | 31 | yes |
| GTP-binding nuclear protein RAN | P62826 | NLQY ^Y DISAK | Y147 | 31 | yes |
| heat shock 70 kDa protein 1A/1B | P08107 | TTPSYVAFTDTER | Y41 | 3 | yes |
| heat shock 70 kDa protein 1A/1B | P08107 | IINEPTAAAIAY ^L GLDR | Y183 | 3 | yes |
| heat shock 70 kDa protein 1A | P0DMV8 | EIAEAY ^L GYPVNTNAVITVPA ^Y FNDSQR | Y134 | nd | no |
| heat shock 70 kDa protein 1A | P0DMV8 | ELEQVCamNPIISGLY ^Q GAGGPGPGGFGAQGPK | Y611 | nd | yes |
| heat shock 70 kDa protein 1A | P0DMV8 | SINPDEAVAY ^G AAVQAAILMoxGDK | Y371 | nd | yes |
| heat shock 70 kDa protein 1A | P0DMV8 | AFY ^P EEISSMVLTK | Y115 | nd | no |
| heat shock protein HSP 90 β | P08238 | FYEAFSK | Y430 | 10 | no |
| heat shock protein HSP 90 β | P08238 | YESLTDPSK | Y56 | 10 | yes |
| heat shock protein HSP 90 β | P08238 | NPDDITQE ^E YGEFYK | Y301 | 10 | yes |
| heat shock protein HSP 90 β | P08238 | NPDDITQE ^E YGEFYK | Y305 | 10 | yes |
| heat shock protein HSP 90 β | P08238 | SIYITGESK | Y484 | 10 | yes |
| 60 kDa heat shock protein, mitochondrial | P10809 | CamEFQDAY ^V LLSEK | Y243 | 69 | yes |
| isoform 2 of heat shock protein HSP 90- α | P07900-2 | DNSTMoxGY ^M oxAAK | Y627 | 58 | yes |
| heterogeneous nuclear ribonucleoproteins A2/B1 | P22626 | DYFEEY ^G K | Y131 | 15 | yes |
| heterogeneous nuclear ribonucleoproteins A2/B1 | P22626 | GGSDGY ^G SGR | Y234 | 15 | no |
| heterogeneous nuclear ribonucleoproteins C1/C2 | P07910 | GFAFVQY ^V NER | Y57 | nd | yes |
| heterogeneous nuclear ribonucleoprotein H2 | P55795 | THYDPPR | Y195 | 784 | no |
| heterogeneous nuclear ribonucleoprotein K | P61978 | AYEPQGGSGYDYSYAGGR | Y361 | 47 | yes |
| isoform 2 heterogeneous nuclear ribonucleoprotein K | P61978-2 | TDYNASVSVDPDSSGPER | Y72 | 47 | yes |
| heterogeneous nuclear ribonucleoprotein L | P14866 | YGGGSGEGGR | Y47 | 95 | yes |
| heterogeneous nuclear ribonucleoprotein L | P14866 | YGGGSGEGGR | Y48 | 95 | yes |
| heterogeneous nuclear ribonucleoprotein U | Q00839 | GYFEYIEENK | Y257 | 117 | yes |
| heterogeneous nuclear ribonucleoprotein U | Q00839 | EKPYFPIPEEYTFIQNVPLEDR | Y466 | 117 | yes |

Table 1. continued

| protein name | access no. | peptide sequence ^b | residue | rank ^c | PhosphoSite ^d |
|---|------------|-------------------------------|---------|-------------------|--------------------------|
| heterogeneous nuclear ribonucleoprotein U | Q00839 | EKPYFPIPEEYTFIQNVPLEDR | Y473 | 117 | yes |
| heterogeneous nuclear ribonucleoprotein U | Q00839 | YNILGNTNTIMoxDK | Y525 | 117 | yes |
| histone H4 | P62805 | ISGLIYEETR | Y51 | 1 | yes |
| histone H4 | P62805 | DAVTYTEHAK | Y72 | 1 | yes |
| histone H4 | P62805 | TVTAMDVVYALK | Y88 | 1 | yes |
| histone H4 | P62805 | TLYGFGG | Y98 | 1 | yes |
| histone H1.4 | P10412 | ALAAAGYDVEK | Y71 | 840 | yes |
| histone H2B type 1L | Q99880 | KESYSVYVYK | Y38 | 6478 | yes |
| histone H1x | Q92522 | YSQLVVETIR | Y48 | 656 | no |
| histone H1.3 | P16402 | ALAAAGYDVEK | Y72 | 4517 | yes |
| L-lactate dehydrogenase A chain | P00338 | DQLIYNLLK | Y10 | 62 | yes |
| L-lactate dehydrogenase A chain | P00338 | QVVESAYEVIK | Y239 | 62 | yes |
| L-lactate dehydrogenase B chain | P07195 | MVVESAYEVIK | Y240 | 20 | yes |
| isoform 2 of NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 11 | Q86Y39 | EKPDDPLNYFLGGCamAGGLTLGAR | Y90 | 789 | no |
| peroxiredoxin-1 | Q06830 | TIAQDYGVLK | Y116 | 9 | yes |
| peroxiredoxin-2 | P32119 | LSEDIYGVLK | Y115 | 50 | yes |
| Phosphoglycerate kinase 1 | P00558 | LGDVYVNDAFGTAHR | Y161 | 102 | yes |
| phosphoglycerate kinase 1 | P00558 | YAEAVTR | Y324 | 102 | no |
| phosphoglycerate kinase 1 | P00558 | YSLEPVAVELK | Y76 | 102 | yes |
| protein NipSnap homologue 3A | Q9UFN0 | SYLKPSPK | Y42 | 3155 | no |
| protein NipSnap homologue 3A | Q9UFN0 | SYLKPSPK | Y43 | 3155 | no |
| pyruvate kinase PKM | P14618 | GDYPLEAVR | Y370 | 42 | yes |
| pyruvate kinase PKM | P14618 | CamDENILWLDYK | Y161 | 42 | yes |
| pyruvate kinase PKM | P14618 | IYVDDGLISLQVK | Y175 | 42 | yes |
| pyruvate kinase PKM | P14618 | EAEAAIYHLQLFEELR | Y390 | 42 | yes |
| pyruvate kinase PKM | P14618 | LNFSHGTHEYHAETIK | Y83 | 42 | yes |
| Ras-related protein Rab-1A | P62820 | YASENVNK | Y112 | 1990 | yes |
| 40S RIBOSOMAL PROTEIN S6 | P62753 | NKEEAAEYAK | Y209 | 114 | yes |
| 40S ribosomal protein S2 | P15880 | SPYQEFTHLVLK | Y266 | 67 | yes |
| 40S ribosomal protein S3a | P61247 | ACamQSIYPLHDVFVR | Y215 | 59 | no |
| 40S ribosomal protein S9 | P46781 | LIGEYGLR | Y35 | 87 | yes |
| 40S ribosomal protein S10 | P46783 | DYHLHPPEIVPATLR | Y82 | 215 | yes |
| 40S ribosomal protein S10 | P46783 | IAIYELLFK | Y12 | 215 | yes |
| 40S ribosomal protein S13 | P62277 | GLAPDLPEDLYHLIK | Y89 | 97 | yes |
| 40S ribosomal protein S19 | P39019 | ELAPYDENWIFYTR | Y48 | 214 | yes |
| 40S ribosomal protein S19 | P39019 | ELAPYDENWIFYTR | Y54 | 214 | yes |
| 60S ribosomal protein L3 | P39023 | IGQGYLIK | Y291 | 46 | yes |
| 60S ribosomal protein L5 | P46777 | NSVTPDMoxMoxEEMoxYK | Y240 | 60 | yes |
| 60S ribosomal protein L6 | Q02878 | YYPTEDEVPR | Y115 | 39 | no |
| 60S ribosomal protein L7 | P18124 | IVEPYIAWGYPNLK | Y139 | 38 | yes |
| 60S acidic ribosomal protein P0 | P05388 | IIQLDDYYPK | Y24 | 135 | yes |
| 60S ribosomal protein L13a | P40429 | YQAVTATLEEK | Y149 | 145 | yes |
| 60S ribosomal protein L23a | P62750 | LYDIDVAK | Y117 | 27 | yes |
| 60S ribosomal protein L27 | P61353 | YSVDIPLDK | Y85 | 32 | no |
| 60S ribosomal protein L29 | P47914 | LAYIAHPK | Y98 | 163 | yes |
| 60S ribosomal protein L36 | Q9Y3U8 | EVCamGFAPYER | Y53 | 82 | no |
| isoform 3 of 60S ribosomal protein L17 | P18621-3 | YSLDPENPTK | Y4 | 164 | yes |
| splicing factor, proline- and glutamine-rich | P23246-1 | NLSPYVSNELLEAFSQFGPIER | Y381 | 263 | yes |
| splicing factor, proline- and glutamine-rich | P23246-1 | YGEPGEVFINIK | Y320 | 263 | yes |
| stress-70 protein, mitochondrial | P38646 | RYDDPEVQK | Y128 | 199 | yes |
| T-complex protein 1 subunit eta | Q99832 | LPIGDVATQYFADR | Y302 | 221 | yes |
| T-complex protein 1 subunit ϵ | P48643 | IADGYEQAAR | Y137 | 127 | no |
| isoform 2 of threonine-tRNA ligase, cytoplasmic | P26639-2 | WELNSGDGAFYGPVK | Y540 | 381 | no |
| isoform 2 of Transgelin-2 | P37802-2 | DGTVLCamELINALYPEGQAPVK | Y70 | 202 | no |
| transmembrane emp24 domain-containing protein 2 | Q15363 | HEQEYMoxEVR | Y150 | 570 | yes |
| tubulin α -1B chain | P68363 | IHFPLATYAPVISAEEK | Y272 | nd | yes |
| tubulin α -1B chain | P68363 | FDGALNVDLTEFQTNLVPPYPR | Y262 | nd | yes |
| tubulin α -1B chain | P68363 | EDAANNYAR | Y103 | nd | yes |
| tubulin β -3 chain | Q13509 | GHYTEGAELVDSVLDDVVR | Y106 | 2533 | yes |
| tubulin β chain | P07437 | YLTVAAVFR | Y310 | 121 | yes |

Table 1. continued

| protein name | access no. | peptide sequence ^b | residue | rank ^c | PhosphoSite ^d |
|--|------------|--|---------|-------------------|--------------------------|
| tubulin β chain | P07437 | NSS Y FVEWIPNNVK | Y340 | 121 | yes |
| tubulin β chain | P07437 | LTTPT Y GDLNHLVSATMoxSGVTTCamLR | Y222 | 121 | yes |
| tubulin β chain | P07437 | ISV Y YNEATGGK | Y50 | 121 | yes |
| tubulin β chain | P07437 | ISV Y YNEATGGK | Y51 | 121 | yes |
| X-ray repair cross-complementing protein 6 | P12956 | NIPP Y FVALVPQEEELDDQK | Y409 | 165 | no |
| 14-3-3 protein ϵ | P62258 | Y LAEFATGNDR | Y131 | 29 | yes |
| 14-3-3 protein ϵ | P62258 | AAFDDAIAELDTLSEES Y K | Y214 | 29 | yes |

^aData were acquired on the Triple-TOF and Orbitrap mass spectrometers. ^bThe labeled residue is shown as bold and underlined. ^cProteins are ranked on the basis of abundance, using iBAQ values for the HEK293 proteome²⁵ (Supplemental Table S4 in Geiger et al.)²⁵ and confirmed in the MaxQB database,²⁶ when possible. Abundance was determined as follows: Proteins in the iBAQ table were arranged in order by iBAQ number, one being the most abundant and 6858 the least abundant. Proteins located in the iBAQ table by their UniProt numbers and their ranks were taken as their positions in the list. iBAQ scoring often combines multiple UniProt numbers into a single value. This occasionally results in the same rank being given to more than one protein. An entry of nd indicates that there was no entry in the iBAQ table for the UniProt number. ^dThe PhosphoSite (<http://www.phosphosite.org/homeAction.action>) was used to check whether the diethoxyphosphorylated tyrosines in this study were also sites for phosphorylation in vivo. Phospho is a yes/no column to indicate if phosphorylation occurs. An entry of nd indicates no data on phosphorylation status for the protein.

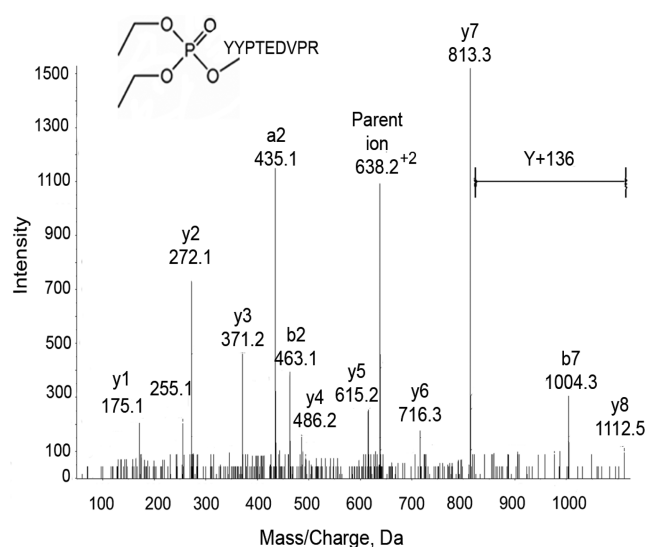


Figure 4. MS/MS spectrum of diethoxyphosphorylated peptide YYPTEDVPR from the 60S ribosomal protein L6 (Q02878). The adduct was on the second tyrosine in the sequence. The added mass on tyrosine from diethoxyphosphate is 136 Da. The difference in mass between y7 and y8 is 299 Da, representing 163 Da from tyrosine and 136 Da from diethoxyphosphate.

consistent with our previous report where we studied selected diethoxyphosphorylated-proteins and peptides.²² Immunopurification on immobilized depY increased the number of diethoxyphospho-tyrosine peptides by 7.7-fold (from 15 to 116), decreased the number of nonlabeled peptides by up to 70-fold (in the Triple TOF study), and excluded diethoxyphosphorylated-lysine. Monoclonal depY binds to diethoxyphosphorylated-tyrosine in peptides independent of the amino acid sequence.²² This means depY could be used to identify modified proteins in any animal including the worm *Caenorhabditis elegans*.³⁰

Tyrosine and Lysine Are More Reactive than Serine in Proteins That Have No Active Site Serine. Our studies with isolated proteins showed that tyrosine and lysine residues are more readily modified by organophosphorus toxicants than serine and threonine.^{17,28,29} The serine hydrolase family of enzymes (acetylcholinesterase, butyrylcholinesterase, and carboxylesterase) is an exception because their active site serine

has an abnormally low pK_a , due to interaction with residues in the catalytic triad that pull a proton off the side-chain hydroxyl group of serine.³¹ Proteins that have no active site serine are more easily modified by nerve agents and organophosphorus pesticides on tyrosine and lysine.^{18,21,32–34} HEK293 cells have no endogenous acetylcholinesterase, butyrylcholinesterase, or carboxylesterase, consistent with the absence of serine adducts in our samples.

Proteins Covalently Modified by CPO on Tyrosine.

One hundred and 16 diethoxyphospho-tyrosine containing peptides on 73 different proteins were identified in HEK293 cell lysates immunopurified with the diethoxyphospho-tyrosine-specific antibody, depY (Table 1). No dealkylated, monoethylphospho-adducts were detected, consistent with previous reports that adducts on tyrosine do not age.^{17,20,21,35} Most of the labeled proteins are in the top 3% of the HEK293 proteome, that is, rank <200 [6858×0.03]. Exceptions to this generalization include calpain small subunit 1 at rank 1390 (20%), actin cytoplasmic 2 at 1710 (25%), tubulin β -3 at 2533 (37%), protein NipSnap homologue 3A at 3155 (46%), histone H1.3 at 4517 (66%), and histone H2B type 1L at 6478 (94%). As expected, the most heavily labeled proteins, that is, proteins with the highest total peptide count, are among the most abundant proteins in the HEK293 proteome: histone H4, heat shock 70 kDa protein 1A/1B, heat shock protein HSP 90 β , and α -enolase.

When the HEK293 cell lysate was treated with 10 μ M CPO, only two diethoxyphospho-tyrosine proteins were detected (histone H4 and histone H1.4). Histone H4 is the most abundant protein in the HEK293 proteome, which could explain its higher reactivity. Histone H1.4 is less abundant, ranking at 12% [$(840 \div 6858) \times 100$], suggesting that one tyrosine in histone H1.4 is especially reactive. A similar special reactivity of one tyrosine in human albumin, Tyr 411, is explained by its location within a pocket near a positively charged residue.^{16,36} In this location the O^4 -phenolic hydroxyl group in tyrosine would be expected to be partly deprotonated allowing the negatively charged residue to displace trichloropyridinol from CPO while simultaneously making a covalent bond with diethoxyphosphate, as shown in Figure 5. The study by Bui-Nguyen et al. with dichlorvos also noted specificity for tyrosines in the vicinity of positively charged arginines or lysines,¹⁸ though nearby amino acids with a hydroxyl group were also found.

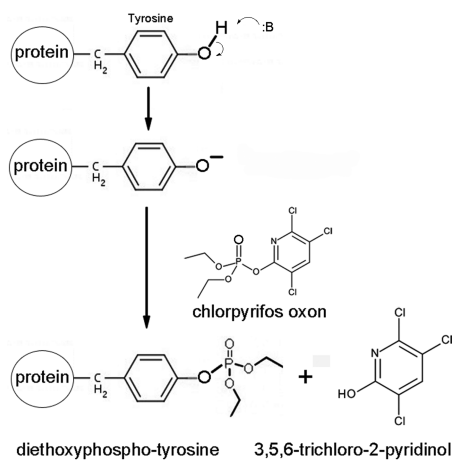


Figure 5. Enhanced reactivity with CPO of a particular tyrosine. High reactivity is explained by the presence of a nearby positively charged residue that stabilizes the ionized phenolic hydroxyl group. The negatively charged side chain of tyrosine attacks the phosphorus atom in chlorpyrifos oxon to form the diethoxyphosphate ester on tyrosine with release of trichloropyridinol.

Physiological Relevance. The CPO concentrations used in our study were not lethal to the cultured cells, but would most likely be lethal to animals and humans. Humans who deliberately poisoned themselves by ingesting chlorpyrifos had up to 12 μM chlorpyrifos in blood at the time of hospital admission 5 h post-ingestion.³⁷ Blood drawn from the same individual over a period of 4 days showed that the CPO concentration in plasma reached a peak of 0.1 μM after 48 h. In another study, the concentration of free CPO in plasma was 0.175 μM for blood drawn 16 h post-ingestion.³⁸ These studies suggest that humans can survive CPO concentrations of 0.175 μM , but are unlikely to survive considerably higher CPO concentrations.

To determine whether a nontoxic dose can form protein adducts in animals, Jiang et al.³⁹ treated mice with 3 mg/kg chlorpyrifos (parent compound; not the oxon) daily for 14 days. The pesticide chlorpyrifos is bioactivated through oxidative desulfuration catalyzed by cytochrome P450 enzymes to form CPO.⁴⁰ Jiang et al.³⁹ found that the mice developed diethoxyphospho adducts on β -tubulin in their brains. The daily dose of 3 mg/kg chlorpyrifos caused no toxicity in the mice and insignificant inhibition of acetylcholinesterase in blood. The diethoxyphosphorylated tubulin was associated with structural modifications of the microtubules as visualized by atomic force microscopy. In order to detect the diethoxyphospho-labeled tubulin, it was necessary to purify the tubulin.

There is no expectation that the same protein adducts will be identified in mouse brain as reported here for human embryonic kidney cells. We anticipate that use of the depY antibody will enrich the diethoxyphospho-tyrosine adducts such that purification of individual proteins will not be necessary and that diethoxyphospho-adducts on unpredicted proteins will be detected.

Reaction with Phospho-Tyrosine Sites. Detergents in the RIPA buffer denatured the solubilized proteins prior to treatment with CPO. The question was raised whether the location of the diethoxyphospho-tyrosine adducts was biologically relevant since the proteins in the cell lysate were in a denatured state before they were treated with CPO. To address the question of biological relevance, we searched the

PhosphoSite (<http://www.phosphosite.org/homeAction.action>), an online systems biology resource providing comprehensive information on protein post-translational modifications including phosphorylation to determine whether the diethoxyphospho-tyrosine sites in the peptides from Table 1 were also phosphorylation sites. Table 1 shows that 96 of the 116 CPO-labeled tyrosines are naturally occurring phosphorylation sites, which means these sites are biologically relevant.

Phosphorylation of tyrosine in proteins by kinases is physiologically relevant and relatively common.⁴¹ Tyrosine phosphorylation and dephosphorylation is a dynamic event that responds within seconds to the needs of a cell.⁴¹ When tyrosine is diethoxyphosphorylated by CPO, the adduct is stable. The modified tyrosine is frozen in a particular state, unable to free itself to serve in signal transduction. We hypothesize that irreversible modification of tyrosines will disrupt homeostasis and could lead to cell death.

Comparison with Another Organophosphorylation Study. The study by Bui-Nguyen et al.¹⁸ was the first to identify organophosphorylated peptides in a complex mixture. Our study is the second. Bui-Nguyen et al. employed dichlorvos (a dimethyl phosphate) to label lysates from the human hepatocyte-like cell line HepaRG. They labeled the lysate with 50, 500, and 5000 μM dichlorvos and acquired data on an Orbitrap Velos mass spectrometer (without enriching the sample). They analyzed the data with the Sequest database search algorithm from Proteome Discoverer and identified 34 nonredundant dimethylphospho-peptide adducts from 29 proteins. Bui-Nguyen et al. found that 26 of the dimethoxyphospho-adducts were on tyrosine, 4 on serine, and 4 on threonine. In our unenriched samples we identified 18 diethoxyphospho-tyrosine containing peptides from 14 proteins as well as 3 diethoxyphospho-lysine adducts, but no diethoxyphospho-serine or diethoxyphospho-threonine adducts. Bui-Nguyen et al. did not look for dimethoxyphospho-lysine adducts. Overall, we found about 2-times fewer peptides and 3-times fewer proteins in our unenriched sample than Bui-Nguyen et al. However, our immunopurified sample identified 116 diethoxyphospho-tyrosine peptides on 73 different proteins, a result that shows the power of using immunopurification to enrich for diethoxyphospho-tyrosine-modified peptides. When we compared our enriched data set to that of Bui-Nguyen et al., 14 peptides in 9 proteins matched. Both studies concluded that organophosphorus toxicants covalently modify tyrosine on a variety of proteins.

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Notes

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the

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ABBREVIATIONS

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BSA, bovine serum albumin; CPO, chlorpyrifos oxon; depY, monoclonal antibody against diethoxyphospho-tyrosine; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; HRP, horseradish peroxidase; iBAQ, intensity-based absolute quantification; LC-MS/MS, liquid chromatography tandem mass spectrometry; PBS, phosphate buffered saline; RIPA, radioimmunoprecipitation assay lysis buffer; TBS, tris buffered saline; TBST, tris buffered saline Tween

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