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

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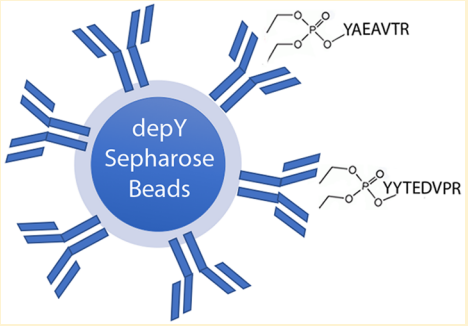
ABSTRACT: Chronic illness from exposure to organophosphorus toxicants is hypothesized to involve modification of unknown proteins. Tyrosine in proteins that have no active site serine readily reacts with organophosphorus toxicants. We developed a monoclonal antibody, depY, that specifically recognizes 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 acetylcholinesterase[2] (AChE) and butyrylcholinesterase[3] (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 AChE[5] 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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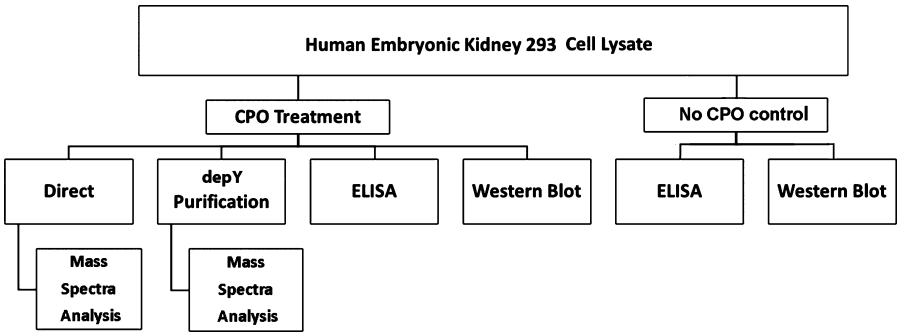

Figure 1. Workflow scheme. Cell lysate with a protein concentration of 4.9 mg/mL was treated with chlorpyrifos oxon (CPO). A portion of the CPO-treated cell lysate was digested with trypsin and analyzed by LC-MS/MS with or without prior immunopurification of tryptic peptides on immobilized monoclonal 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 cresylphospho-serine.

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 14,000*g*. 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 μg/100 μL 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

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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 μL of 3,3′,5,5′-tetramethylbenzidine per well. The HRP reaction was stopped after 20 min by addition of 100 μL 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 μM 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 μL beads covalently bound to 30 μg of depY.

Sample Preparation for Mass Spectrometry. The sample preparation method was adapted from Wisniewski et al.[23] Disulfide bonds in cell lysates (380 μg 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 μL). These solutions were vortexed and heated in a boiling water bath for 3 min. Each sample was mixed with 200 μL 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. MRCPRT010), and centrifuged at 14,000*g* for 10 min until the sample compartment was nearly dry. Excess dithiothreitol was washed off with 400 μL of 8 M urea, centrifuged at 14,000*g* for 15 min until the filter was nearly dry. This process was repeated twice. Sulfhydryl groups were carbamidomethylated by adding 100 μL 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 14,000*g* for 10 min, followed by two washes with 100 μL of 8 M urea. The contents of each filter unit were equilibrated with 100 μL of 50 mM ammonium bicarbonate pH 8 and centrifuged at 14,000*g* for 10 min. This step was repeated twice. The contents of each filter were suspended in 100 μL of 50 mM ammonium bicarbonate and mixed with 10 μL of 0.4 mg/mL trypsin (trypsin to protein ratio about ∼1:100 μg/μg). Tryptic digestion was performed on the filter, in a humidified chamber, at 37 °C, overnight (16−18 h). Filter units were centrifuged at 14,000*g* for 10 min into new tubes to collect the tryptic peptides, rinsed with 100 μL of 0.5 M sodium chloride, and centrifuged at 14,000*g* 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 μL of 28% ammonium hydroxide plus 950 μL 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 μL of 0.1% formic acid (unenriched samples, about 10 μg protein per μL 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 μL of depY Sepharose beads in PBS for 2 h. Beads were placed in a 0.45 μm 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 μL of 0.1% formic acid for LC-MS/MS analysis (enriched sample, <10 μg protein per μL).

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 μg or less of sample, in a 5 μL 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 C_{18} (3 μm, 120 Å particles; Trap: 200 μm × 0.5 mm; Separation: 75 μm × 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 μL/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 μL/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 5.5[24] 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 = organo-phosphylation, species = *Homo sapiens*, ID focus = biological modifications, database = uniprot_sprotJAN2015.fasta, search effort = thorough, and FDR analysis = active. A new section entitled organophosphylation 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

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UHPLC (Dionex, Sunnyvale, CA). Chromatography employed an Acclaim PepMap RSLC C18 column (75 μm × 150 mm) (Thermo Scientific no. 164534) and an Acclaim PepMap 100 trap column (75 μm × 20 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 μL/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 μL/min.

The OrbiTrap Fusion Lumos was fitted with a Nanospray Flex NG ion source (Thermo Scientific) and a stainless steel Nanobore emitter (Thermo Finnigan #ES542). 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 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.

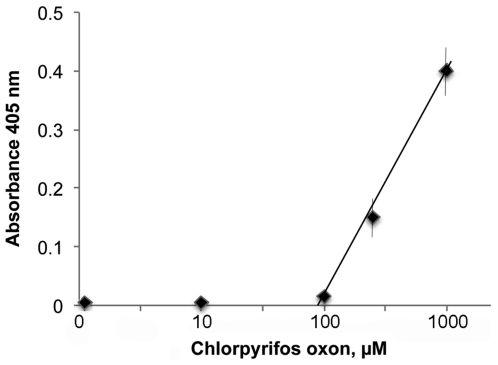

Figure 2. ELISA analysis of the interaction of monoclonal antibody depY with CPO-labeled proteins in HEK293 cell lysates treated with 0, 10, 100, 250, or 1000 μM CPO. The depY antibody concentration was 0.02 μg in 100 μL of 1% BSA/TBS. Data points are the mean of four measurements ± SD. Data were corrected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

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

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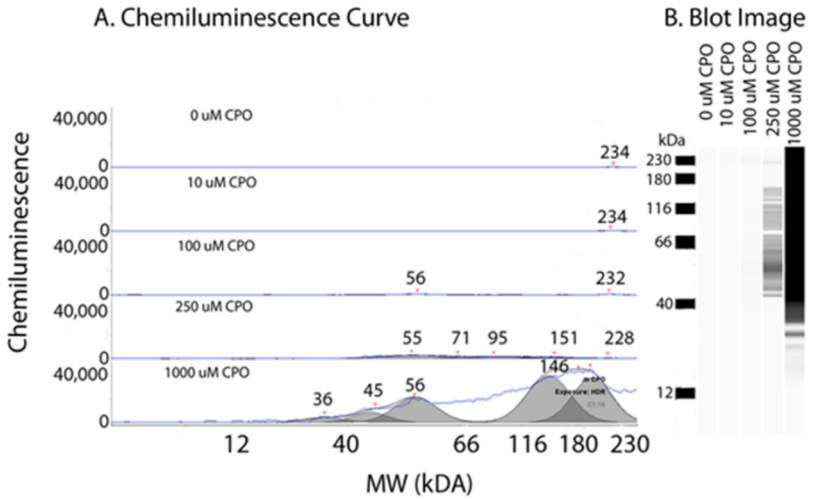

Figure 3. Capillary electrophoresis-based Western blot analysis (RayBiotech) of the interaction of monoclonal antibody, depY, with diethoxyphospho-tyrosine-labeled proteins from HEK293 cell lysates. Samples containing 0.008 μg protein in 40 nL were hybridized with 0.02 mg/mL monoclonal antibody depY. (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 ÷ 44,921 × 100]. With depY extraction, 17% of the peptides were diethoxyphosphorylated [109 ÷ 629 × 100], a 280-fold enhancement [17 ÷ 0.06]. The total number of peptides in the depY extracted sample was 70-fold lower than in the unextracted sample [44,921 ÷ 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 y8 ion. If the adduct had been on the N-terminal tyrosine, the y8 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 tyrosine[28] and lysine[29] 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

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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	DS**Y**VGDEAQSK	Y53	1710	yes
actin, cytoplasmic 2	P63261	DLTD**Y**LMK	Y188	1710	yes
actin, cytoplasmic 2	P63261	G**Y**SFTTTAER	Y198	1710	yes
actin, cytoplasmic 2	P63261	QE**Y**DESGPSIVHR	Y362	1710	yes
actin, cytoplasmic 2	P63261	S**Y**ELPDGQVITIGNER	Y240	1710	yes
actin, cytoplasmic 2	P63261	DL**Y**ANTVLSGGTTMYPGIADR	Y294	1710	yes
actin, cytoplasmic 2	P63261	IWHHTF**Y**NELR	Y91	1710	yes
actin, α cardiac muscle 1	P68032	**Y**PIEHGIITNWDDMoxEK	Y71	156	yes
ADP/ATP translocase 2	P05141	**Y**FPTQALNFAFK	Y81	101	yes
poly [ADP-ribose] polymerase 1	P09874	F**Y**TLIPHDFGMoxK	Y737	151	yes
adenine phosphoribosyl transferase	P07741-1	ID**Y**IAGLDSR	Y60	392	yes
α-enolase	P06733	AAVPSGASTGI**Y**EALELR	Y44	14	yes
α-enolase	P06733	IGAEV**Y**HNLK	Y189	14	yes
α-enolase	P06733	AG**Y**TDKVVIGMDVAASEFFR	Y236	14	yes
α-enolase	P06733	**Y**ISPDQLADLYK	Y270	14	yes
α-enolase	P06733	D**Y**PVVSIEDPFDQDDWGAWQK	Y287	-	yes
ATP synthase subunit α, mitochondrial	P25705-1	EA**Y**PGDVFYLHSR	Y337	225	yes
ATP synthase subunit α, mitochondrial	P25705-1	EAYPGDVF**Y**LHSR	Y343	225	yes
ATP synthase subunit β, mitochondrial	P06576	IMDPNIVGSEH**Y**DVAR	Y418	141	yes
ATP synthase subunit γ, mitochondrial	P36542-1	I**Y**GLGSLALYEK	Y69	637	no
calpain small subunit 1	P04632	**Y**SDESGNMoxDFDNFISCamLVR	Y217	1390	no
cofilin-1	P23528	EILVGDVGQTVDDP**Y**ATFVK	Y68	25	yes
cofilin-1	P23528	HELQANC**Y**EEVK	Y140	25	yes
cofilin-1	P23528	**Y**ALYDATYETK	Y82	25	yes
cofilin-1	P23528	YAL**Y**DATYETK	Y85	25	yes
cofilin-1	P23528	YALYDAT**Y**ETK	Y89	25	yes
elongation factor 1-α 1	P68104	**Y**YVTIIDAPGHR	Y85	8	yes
elongation factor 1-α 1	P68104	Y**Y**VTIIDAPGHR	Y86	8	yes
elongation factor 1-α 1	P68104	EHALLA**Y**TLGVK	Y141	8	yes
elongation factor 1-α 1	P68104	IG**Y**NPDTVAFVPISGWNGDNMoxLEPSANMoxPWFK	Y183	8	no
elongation factor 2	P13639	**Y**FDPANGK	Y265	26	yes
Isoform 2 of elongation factor 1-γ	P26641-2	ILGLLDA**Y**LK	Y145	100	yes
putative elongation factor 1-α-like 3	Q5VTE0	CVESFSD**Y**PPLGR	Y418	8	nd
endoplasmin	P14625	DISTNY**Y**ASQK	Y678	228	yes
glyceraldehyde-3-phosphate dehydrogenase	P04406-1	LISW**Y**DNEFGYSNR	Y314	12	yes
glyceraldehyde-3-phosphate dehydrogenase	P04406-1	LISWYDNEFG**Y**SNR	Y320	12	yes
GTP-binding nuclear protein RAN	P62826	NLQ**Y**YDISAK	Y146	31	yes
GTP-binding nuclear protein RAN	P62826	NLQY**Y**DISAK	Y147	31	yes
heat shock 70 kDa protein 1A/1B	P08107	TTPS**Y**VAFTDTER	Y41	3	yes
heat shock 70 kDa protein 1A/1B	P08107	IINEPTAAAIA**Y**GLDR	Y183	3	yes
heat shock 70 kDa protein 1A	P0DMV8	EIAEA**Y**LGYPVTNAVITVPA**Y**FNDSQR	Y134	nd	no
heat shock 70 kDa protein 1A	P0DMV8	ELEQVCamNPIISGL**Y**QGAGGPGPGGFGAQGPK	Y611	nd	yes
heat shock 70 kDa protein 1A	P0DMV8	SINPDEAVA**Y**GAAVQAAILMoxGDK	Y371	nd	yes
heat shock 70 kDa protein 1A	P0DMV8	AF**Y**PEEISSMVLTK	Y115	nd	no
heat shock protein HSP 90 β	P08238	F**Y**EAFSK	Y430	10	no
heat shock protein HSP 90 β	P08238	**Y**ESLTDPSK	Y56	10	yes
heat shock protein HSP 90 β	P08238	NPDDITQEE**Y**GEFYK	Y301	10	yes
heat shock protein HSP 90 β	P08238	NPDDITQEEYGEF**Y**K	Y305	10	yes
heat shock protein HSP 90 β	P08238	SI**Y**YITGESK	Y484	10	yes
60 kDa heat shock protein, mitochondrial	P10809	CamEFQDA**Y**VLLSEK	Y243	69	yes
isoform 2 of heat shock protein HSP 90-α	P07900-2	DNSTMoxG**Y**MoxAAK	Y627	58	yes
heterogeneous nuclear ribonucleoproteins A2/B1	P22626	D**Y**FEEYGK	Y131	15	yes
heterogeneous nuclear ribonucleoproteins A2/B1	P22626	GGSDG**Y**GSGR	Y234	15	no
heterogeneous nuclear ribonucleoproteins C1/C2	P07910	GFAFVQ**Y**VNER	Y57	nd	yes
heterogeneous nuclear ribonucleoprotein H2	P55795	TH**Y**DPPR	Y195	784	no
heterogeneous nuclear ribonucleoprotein K	P61978	A**Y**EPQGGSGYDYSYAGGR	Y361	47	yes
isoform 2 heterogeneous nuclear ribonucleoprotein K	P61978-2	TD**Y**NASVSVPDSSGPER	Y72	47	yes
heterogeneous nuclear ribonucleoprotein L	P14866	**Y**YGGGSEGGR	Y47	95	yes
heterogeneous nuclear ribonucleoprotein L	P14866	Y**Y**GGGSEGGR	Y48	95	yes
heterogeneous nuclear ribonucleoprotein U	Q00839	G**Y**FEYIEENK	Y257	117	yes
heterogeneous nuclear ribonucleoprotein U	Q00839	EKP**Y**FPIPEEYTFIQNVPLEDR	Y466	117	yes

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Table 1. continued

protein name	access no.	peptide sequence[b]	residue	rank[c]	PhosphoSite[d]
heterogeneous nuclear ribonucleoprotein U	Q00839	EKPYFPIPEE**Y**TFIQNVPLEDR	Y473	117	yes
heterogeneous nuclear ribonucleoprotein U	Q00839	**Y**NILGTNTIMoxDK	Y525	117	yes
histone H4	P62805	ISGLI**Y**EETR	Y51	1	yes
histone H4	P62805	DAVT**Y**TEHAK	Y72	1	yes
histone H4	P62805	TVTAMDVV**Y**ALK	Y88	1	yes
histone H4	P62805	TL**Y**GFGG	Y98	1	yes
histone H1.4	P10412	ALAAAG**Y**DVEK	Y71	840	yes
histone H2B type 1L	Q99880	KES**Y**SVYVYK	Y38	6478	yes
histone H1x	Q92522	**Y**SQLVVETIR	Y48	656	no
histone H1.3	P16402	ALAAAG**Y**DVEK	Y72	4517	yes
L-lactate dehydrogenase A chain	P00338	DQLI**Y**NLLK	Y10	62	yes
L-lactate dehydrogenase A chain	P00338	QVVESA**Y**EVIK	Y239	62	yes
L-lactate dehydrogenase B chain	P07195	MVVESA**Y**EVIK	Y240	20	yes
isoform 2 of NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 11	Q86Y39	EKPDDPLN**Y**FLGGCamAGGLTLGAR	Y90	789	no
peroxiredoxin-1	Q06830	TIAQD**Y**GVLK	Y116	9	yes
peroxiredoxin-2	P32119	LSED**Y**GVLK	Y115	50	yes
Phosphoglycerate kinase 1	P00558	LGDV**Y**VNDAFGTAHR	Y161	102	yes
phosphoglycerate kinase 1	P00558	**Y**AEAVTR	Y324	102	no
phosphoglycerate kinase 1	P00558	**Y**SLEPVAVELK	Y76	102	yes
protein NipSnap homologue 3A	Q9UFN0	S**Y**YLKPSK	Y42	3155	no
protein NipSnap homologue 3A	Q9UFN0	SY**Y**LKPSK	Y43	3155	no
pyruvate kinase PKM	P14618	GD**Y**PLEAVR	Y370	42	yes
pyruvate kinase PKM	P14618	CamDENILWLD**Y**K	Y161	42	yes
pyruvate kinase PKM	P14618	I**Y**VDDGLISLQVK	Y175	42	yes
pyruvate kinase PKM	P14618	EAEAAI**Y**HLQLFEELR	Y390	42	yes
pyruvate kinase PKM	P14618	LNFSHGTHE**Y**HAETIK	Y83	42	yes
Ras-related protein Rab-1A	P62820	**Y**ASENVNK	Y112	1990	yes
40S RIBOSOMAL PROTEIN S6	P62753	NKEEAAE**Y**AK	Y209	114	yes
40S ribosomal protein S2	P15880	SP**Y**QEFTDHLVK	Y266	67	yes
40S ribosomal protein S3a	P61247	ACamQSI**Y**PLHDVFVR	Y215	59	no
40S ribosomal protein S9	P46781	LIGE**Y**GLR	Y35	87	yes
40S ribosomal protein S10	P46783	D**Y**LHLPPEIVPATLR	Y82	215	yes
40S ribosomal protein S10	P46783	IAI**Y**ELLFK	Y12	215	yes
40S ribosomal protein S13	P62277	GLAPDLPEDL**Y**HLIK	Y89	97	yes
40S ribosomal protein S19	P39019	ELAP**Y**DENWFYTR	Y48	214	yes
40S ribosomal protein S19	P39019	ELAPYDENWF**Y**TR	Y54	214	yes
60S ribosomal protein L3	P39023	IGQG**Y**LIK	Y291	46	yes
60S ribosomal protein L5	P46777	NSVTPDMoxMoxEEMox**Y**K	Y240	60	yes
60S ribosomal protein L6	Q02878	Y**Y**PTEDVPR	Y115	39	no
60S ribosomal protein L7	P18124	IVEP**Y**IAWGYPNLK	Y139	38	yes
60S acidic ribosomal protein P0	P05388	IIQLLDD**Y**PK	Y24	135	yes
60S ribosomal protein L13a	P40429	**Y**QAVTATLEEK	Y149	145	yes
60S ribosomal protein L23a	P62750	L**Y**DIDVAK	Y117	27	yes
60S ribosomal protein L27	P61353	**Y**SVDIPLDK	Y85	32	no
60S ribosomal protein L29	P47914	LA**Y**IAHPK	Y98	163	yes
60S ribosomal protein L36	Q9Y3U8	EVCamGFAP**Y**ER	Y53	82	no
isoform 3 of 60S ribosomal protein L17	P18621-3	**Y**SLDPENPTK	Y4	164	yes
splicing factor, proline- and glutamine-rich	P23246-1	NLSP**Y**VSNELLEEAFSQFGPIER	Y381	263	yes
splicing factor, proline- and glutamine-rich	P23246-1	**Y**GEPGEVFINK	Y320	263	yes
stress-70 protein, mitochondrial	P38646	R**Y**DDPEVQK	Y128	199	yes
T-complex protein 1 subunit eta	Q99832	LPIGDVATQ**Y**FADR	Y302	221	yes
T-complex protein 1 subunit ε	P48643	IADG**Y**EQAAR	Y137	127	no
isoform 2 of threonine-tRNA ligase, cytoplasmic	P26639-2	WELNSGDGAF**Y**GPK	Y540	381	no
isoform 2 of Transgelin-2	P37802-2	DGTVLCamELINAL**Y**PEGQAPVK	Y70	202	no
transmembrane emp24 domain-containing protein 2	Q15363	HEQE**Y**MoxEVR	Y150	570	yes
tubulin α-1B chain	P68363	IHFPLAT**Y**APVISAEK	Y272	nd	yes
tubulin α-1B chain	P68363	FDGALNVDLTEFQTNLVP**Y**PR	Y262	nd	yes
tubulin α-1B chain	P68363	EDAANN**Y**AR	Y103	nd	yes
tubulin β-3 chain	Q13509	GH**Y**TEGAELVDSVLDVVR	Y106	2533	yes
tubulin β chain	P07437	**Y**LTVAAVFR	Y310	121	yes

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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	ISVY**Y**NEATGGK	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

[a]Data were acquired on the Triple-TOF and Orbitrap mass spectrometers. [b]The labeled residue is shown as bold and underlined. [c]Proteins are ranked on the basis of abundance, using iBAQ values for the HEK293 proteome[25] (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. [d]The 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.

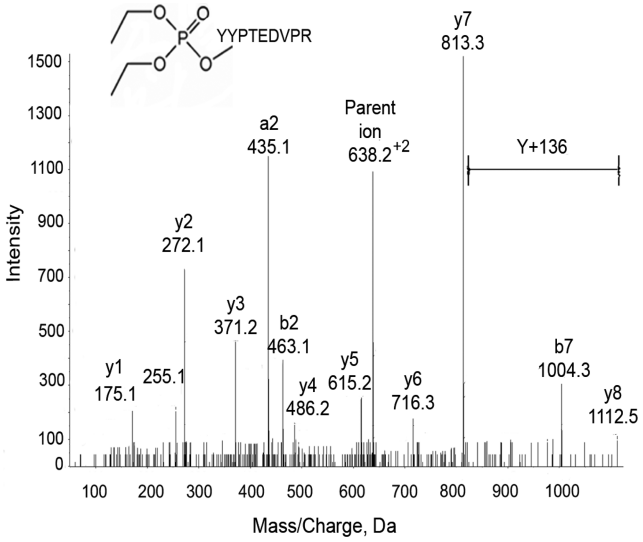

Figure 4. MS/MS spectrum of diethoxyphosphorylated peptide YYPTEDVPR from the 60S ribosomal protein L6 (Q02878). The adduct was on the second tyrosine in the sequence. The added mass on tyrosine from diethoxyphosphate is 136 Da. The difference in mass between y7 and y8 is 299 Da, representing 163 Da 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 μM 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 ÷ 6858) × 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.

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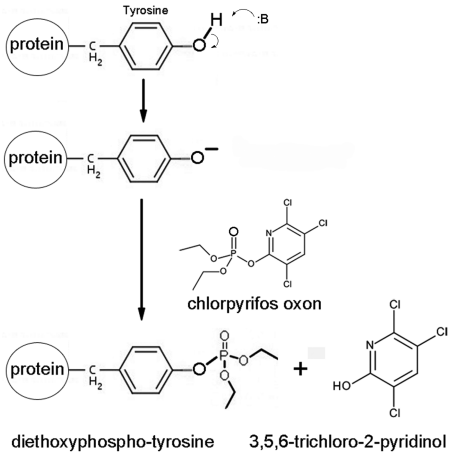

Figure 5. Enhanced reactivity with CPO of a particular tyrosine. High reactivity is explained by the presence of a nearby positively charged residue that stabilizes the ionized phenolic hydroxyl group. The negatively charged side chain of tyrosine attacks the phosphorus atom in chlorpyrifos oxon to form 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 doşe 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 diethylphospho-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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Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

The authors declare no competing financial interest.

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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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