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A Proteomic Approach to Identification of Plutonium Binding Proteins in Mammalian Cells

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Abstract

Plutonium can enter the body through different routes and remains there for decades; however its specific biochemical interactions are poorly defined. We, for the first time, have studied plutonium-binding proteins using a metalloproteomic approach with rat PC12 cells. A combination of immobilized metal ion chromatography, 2D gel electrophoresis, and mass spectrometry were employed to analyze potential plutonium-binding proteins. Our results show that several proteins from PC12 cells show affinity towards Pu⁴⁺-NTA (plutonium bound to nitrilotriacetic acid). Proteins from seven different spots in the 2D gel were identified. In contrast to the previously known plutonium-binding proteins transferrin and ferritin, which bind ferric ions, most identified proteins in our experiment are known to bind calcium, magnesium, or divalent transition metal ions. The identified plutonium interacting proteins also have functional roles in downregulation of apoptosis and other pro-proliferative processes. MetaCore analysis based on this group of proteins produced a pathway with a statistically significant association with development of neoplastic diseases.

Keywords

PC12 cells; 2-D gel electrophoresis; proteomics; plutonium-binding proteins; IMAC; anti-apoptotic; GO process

INTRODUCTION

Plutonium is a toxic synthetic element produced in nuclear reactors. It has no useful biological function. There are currently more than 1000 metric tons of plutonium in the world, and the chances of accidental exposure to plutonium or other synthetic actinide elements is increasing with the increasing worldwide reliance on nuclear energy. Understanding how to limit the biological hazards of plutonium depends on understanding its interaction with the biological environment. Consequently, the biology of plutonium has been of interest since the element was discovered in the 1940s. Many workers have studied

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Supporting Information Available:

2D gel image for proteins that showed affinity for Ho³⁺-NTA resin (Figure S1), 2D gel image for cell extract eluted from NTA resin in the absence of metal (Figure S2), and a list of proteins, their most frequent subcellular locations and the number of edges they were involved in in the schematic pathway (Table S1). This material is available free of charge at <http://pubs.acs.org>.

the distribution of plutonium in organs and tissues¹⁻³ but most of these studies were focused on concentration measurements, and few specific molecular level details are understood. This lack of knowledge about plutonium biochemistry has hindered development of agents for the selective remediation and decorporation of plutonium or other actinides.⁴⁻⁶ Achieving a molecular level understanding of the uptake and distribution of plutonium and its biochemical activity will require identification of the essential biomolecules (proteins or small molecules) involved in the intracellular plutonium trafficking and sequestration.

Plutonium can exist in four different oxidation states (Pu(III), Pu(IV), Pu(V), and Pu(VI)) under normal environmental conditions.^{7, 8} Pu(IV) has been of particular interest due to its relative stability in cells as shown by XANES measurement.⁹ In the absence of potent ligands Pu(IV) is strongly hydrolyzed to form polymeric species at physiological pH,¹⁰⁻¹² however plutonium polymers are not readily taken up by mammalian cells.¹³ Therefore, some biological molecules (proteins, nucleic acids, or small molecules) must stabilize and sequester plutonium in living organisms.¹⁴ Being a synthetic, nonessential element, no such proteins have naturally evolved to selectively bind and sequester plutonium. Consequently, plutonium binding to proteins are strictly adventitious. An important example of this is that Pu(IV) partially follows Fe(III) biochemistry, associating with the extracellular iron transport protein transferrin and the intracellular iron storage protein ferritin.¹⁴ In addition to the known transferrin-mediated iron uptake pathway,¹⁵ plutonium likely also follows other pathways to enter cells.¹⁶ Regardless of the pathways involved, however, it is not understood how plutonium moves from the extracellular milieu to ferritin inside cells. In addition, although plutonium is a well known carcinogen, its carcinogenicity is considered to arise as an effect of its ionizing radiation. The possible role of plutonium in stimulating neoplasia through chemical effects has not been elucidated yet.

The liver and skeleton are the major deposition sites for intravenously injected plutonium with lesser amounts depositing in the spleen, kidneys, lung, testes, and lymph nodes.^{3, 17} In certain instances, the plutonium concentration in the adrenal glands has been reported to be even higher than in the skeleton or liver.^{18, 19} Being much smaller than the liver or skeleton, the adrenal glands are unlikely to be important in determining the whole body radiation dose, but studying them can provide critical molecular level information about the speciation and mechanisms of intracellular plutonium transport. Therefore, we selected PC12 cell lines from the rat adrenal gland in this study to understand the interactions of plutonium with intracellular proteins.

One third of known proteins are metalloproteins, and many metalloproteomes in both prokaryotes and eukaryotes have not been fully characterized.²⁰ Proteomic analysis of PC12 cells shows more than 1000 protein spots in the gel.²¹ It is challenging to select target metalloproteins from the thousands of proteins present in a cell, however metalloproteomics can be used to identify metal specific proteins using immobilized metal affinity chromatography (IMAC), 2D gel electrophoresis (2DE), and mass spectrometry.²²⁻²⁵ Proteomics of uranyl,²² bismuth,²³ and several transition metal ions²⁶ have been studied in bacterial or mammalian cells using this approach. Nickel bound nitrilotriacetic acid (NTA) functionalized sepharose resin is commonly used for purification of histidine-tagged proteins. NTA is also a good chelating agent for Pu⁴⁺,²⁷ therefore, we selected NTA functionalized sepharose to capture plutonium binding proteins. Our results show that several proteins from PC12 cells interact strongly with Pu⁴⁺-NTA and are separated by 2DE. The proteins we identified from LC-MS/MS have functional roles in anti-apoptotic processes and cell substrate adhesion and are thus involved in pro-proliferation with possible roles in cancer development. Most of these proteins also show metal binding properties. Thus the association of plutonium with these proteins may stimulate neoplasia by chemical means.

Experimental

Safety Precaution

All isotopes of plutonium are alpha-emitting radionuclides that must be handled in specially designed radiological containment.

Cell Culture

PC12 cells (American Type Culture Collection, Manassas, VA, USA) were grown in F12K media supplemented with 12.5% serum, 2.5% fetal calf serum and antibiotics in humidified 5% CO₂ environment at 37 °C. Cells were plated in 50 cm² culture bottles to about 70% confluency. Cells were harvested and either lysed immediately in native lysis buffer (10 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, pH 7.4, 1 mM phenylmethanesulfonylfluoride) or stored at –80 °C and lysed later when needed. After incubation in lysis buffer for 40 minutes at 4 °C, the cell suspension was centrifuged for 30 minutes at 4 °C. The clear supernatant was syringe filtered through a 0.22 µm filter. The protein concentration was estimated by BCA assay. Approximately 1–2 mg of protein from the cell lysate was loaded onto a 0.5 mL Pu⁴⁺-NTA column for IMAC.

Preparation of the Pu⁴⁺-NTA Column

To reduce radiological effects, the isotope ²⁴²Pu ($t_{1/2} = 3.76 \times 10^5$ years) was used in all experiments unless otherwise indicated. A 0.5 mL NTA superflow (QIAGEN Inc.) column was prepared at room temperature. Pu³⁺ was prepared in 1M perchloric acid solution by electrolytic reduction as described earlier.¹³ The perchloric acid concentration was lowered to about 0.1 M with four contacts of 10% Amberlite-LA in *o*-xylene that was 90% of the Pu³⁺ solution by volume. The remaining HClO₄ in the Pu³⁺ solution was neutralized with solid Na₂CO₃ to pH ~ 2 and then the blue Pu solution was loaded onto the NTA column. Plutonium binding was indicated by the color of the resin changing from white to blue with a colorless effluent. After 10–20 minutes of incubation, unbound plutonium was washed away at pH 2 and the pH of the column buffer was raised slowly to about 6. The column was left at room temperature one hour to allow air oxidation of the Pu³⁺ to Pu⁴⁺. Complete oxidation was confirmed by color change from blue to light pink. Protein binding to the Pu-NTA column was tested using bovine serum transferrin, a high affinity plutonium binding protein.

Protein Capture by the Pu⁴⁺-NTA Column

After loading, the Pu⁴⁺-NTA column was further washed and equilibrated with ice cold 10 mM HEPES, 0.1 M NaCl (pH 7.0). 1 mL of cell lysate containing 1–2 mg/mL protein was passed through the column twice, and then the column was incubated at 4 °C for 10 minutes. Unbound proteins from the column were washed with 15 column volumes of ice cold HEPES buffer. Bound proteins were eluted from the column with 0.1 M acetate, 0.1 M NaCl, 0.5 M imidazole at pH 4.5. EDTA or other chelating agents were not used in the elution buffer to avoid plutonium contamination of the proteins in order to make protein characterization easier. Eluted proteins were then spin-dialyzed using 10 mM Tris-HCl at pH 7.5 to reduce the salt concentration. The eluted proteins were further desalted using ice cold acetone at twice the volume of protein solution. After incubation for 3 hours at 4 °C, the resulting solution was centrifuged for 30 minutes at 13000 rpm. The supernate was discarded, and the remaining protein pellet was air dried and dissolved in 200 µL of rehydration buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lytes). 185 µL of this rehydration buffer with protein was used to rehydrate 11 cm IPG strips (Bio-Rad) overnight at room temperature. IPG strips were then subjected to isoelectric focusing (IEF) at 20 °C. After the completion of the IEF run, the IPG strips were equilibrated with buffer I (6 M urea,

2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 2% (w/v) DTT) and buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol and 2.5% (w/v) iodoacetamide) for 10 minutes each. The strips were then subjected to 12% second dimensional SDS-PAGE. SDS-PAGE was performed for one hour at 185V. After fixing the gel in 40% ethanol, 10% acetic acid, protein spots were visualized using a mass spectrometry compatible silver staining protocol.²⁸ Similar experiments were repeated using Fe³⁺-NTA, Ho³⁺-NTA and metal-free NTA resin. No proteins were found to bind the metal-free NTA resin (Supplementary Figure S2).

Protein Identification

The protein spots from multiple runs were tested for the presence of plutonium using alpha spectrometry. No radioactivity from plutonium was detected above background levels. Protein spots were excised from the gel and stored at -20 °C until use. The protein samples were trypsin digested and analyzed by LC-MS/MS using a LTQ-Orbitrap Velos at the Donald Danforth Plant Science Center, Proteomics & Mass Spectrometry Facility (St. Louis, MO). Protein identification is based on the criteria of matching at least two rat peptide sequences per sample with at least 10% sequence coverage, greater than 80% probability for each peptide, and a mascot ion score higher than 30.²⁹ The data were searched against the NCBIInr database using mascot as a search engine. Based on these peptides, proteins identified with greater than 99.00% probability have been reported. Proteins from gel spots not adhering to all the criteria given above were not considered. The percentage of sequence coverage and molecular mass were given high priority in assigning proteins to the particular spots if the same protein showed probability in multiple spots.

MetaCore Analysis

Among the Pu-binding proteins identified by 2DE and mass spectrometry the five proteins confirmed with highest confidence (at least 4 peptides or more) were subjected to analysis with MetaCore version 6.2 (GeneGo, Inc. Thomson Reuters; St. Joseph, MI). The MetaCore database contains curated biological molecule relationships for millions of proteins and small molecules, based on published literature. The relationships include every possible combination of direct protein interactions, transcriptional and post-transcriptional, translational and post-translational regulation, ligand-receptor binding, enzyme-substrate interactions, etc. The GeneGo "Shortest Paths" Pathway Maps algorithms were used to create a hypothetical networks of Pu binding proteins; the algorithm included additional proteins required to create a network based on the curated interactions. The ultimate gene network had only those edges that participate in the shortest protein interaction paths.

RESULTS AND DISCUSSIONS

Identification of Pu-Binding Proteins

Native PAGE is used to separate proteins in their native form where protein mobility depends on charge density, amino acid composition, post-translation modifications as well as the compactness of the folded conformation. Therefore all proteins may not move in the gel under the same conditions giving poor gel resolution. An attempt to directly screen Pu-binding proteins by tracking the plutonium (²³⁸Pu) radioactivity in native gel electrophoresis failed because of the plutonium ion dissociated from the proteins during the electrophoresis and staining/de-staining processes.³⁰ Even for the well-known high affinity Pu-binding protein, transferrin (with a reported conditional stability constant of Pu-Tf, $\log \beta = 21.25 \pm 0.75$),¹⁴ less than 1% of the loaded Pu was found associated with transferrin after the completion of native 2D gel electrophoresis. Most plutonium was lost from the proteins either during IEF due to the high applied voltage (8000 V) or by denaturation during de-staining process in 10% acetic acid, 40% methanol.

Because we could not directly identify which proteins from the whole cell lysate bound plutonium, we attempted to separate plutonium binding proteins on a Pu⁴⁺-NTA column followed by 2D gel electrophoresis to isolate the proteins that bind plutonium. This approach has the added advantage that the plutonium remains on the NTA column so the plutonium-free protein spots can be analyzed by conventional peptide mass fingerprinting without equipment dedicated to measuring radioactive samples. The efficacy of this Pu-NTA IMAC approach was also tested using transferrin, which is known to bind Pu⁴⁺-NTA complexes.³¹

Proteins isolated from the Pu⁴⁺-NTA column in their native state were subjected to first and second dimensional gels under denaturing and reducing conditions. From 2D gel electrophoresis several protein spots (about 20 spots) were visualized in the gel by silver staining (Figure 1). Eleven of the most abundant protein spots that appeared in more than three experiments were selected for mass analysis. Of these 11 spots, proteins from seven spots were identified with 100 % probability from peptide mass and sequence matching to rat proteins (Table 1). All identified proteins, their accession numbers, and functions including metal binding properties and selected GO (gene ontology) processes are listed in supplementary Table 1. The GO terms for these proteins were taken from GO database on August 17, 2011 (<http://amigo.geneontology.org/cgi-bin/amigo/search.cgi>).

Plutonium shows certain chemical similarities to lanthanides and Fe³⁺. Therefore, to understand if the proteins isolated using the Pu-NTA column were unique to Pu, similar experiments were conducted substituting Fe³⁺ or the lanthanide cation Ho³⁺ for Pu⁴⁺ on the NTA column. When the lanthanide holmium was substituted for Pu, the proteins appearing in the 2D gels (Supplementary Material Figure S1) were mostly different from the proteins isolated from Pu-NTA resin. Only three proteins appeared in the same locations, (the plutonium-based experiments identified these as GRP78, Galectin-1, and rCG57843 isoform CRA_a, which is identical to M2 pyruvate kinase). Galectin-1 and two other unidentified proteins were observed in the same locations in gels for both Pu⁴⁺ and Fe³⁺-NTA binding proteins. It should be noted that the identities of the Ho³⁺-NTA and Fe³⁺-NTA binding proteins were not confirmed by LC-MS/MS analysis. This result indicates that the isolated proteins are from the specific interaction with the metal-NTA complexes on the resin and not simply from hydrophobic or ionic interactions with resin itself.

Metal Binding and Biochemical Properties of Identified Proteins

Among the identified proteins, 78 kDa glucose-regulated protein (GRP78), nucleoside diphosphate kinase B, and actin have previously been shown to bind another actinide, the hexavalent uranyl cation UO₂²⁺, as well as other metal ions.^{22, 32} GRP78 is identified as spot 1 in the Pu-NTA samples. It is a member of the 70-kDa heat shock proteins (Hsp70). It naturally binds one Mg²⁺-ATP or Ca²⁺-ATP complex with four additional water molecules coordinating to the Mg²⁺ or Ca²⁺ cation. In addition to the Ca²⁺ ion in its nucleotide binding site, the crystal structure of human GRP78 also shows a second Ca²⁺ ion bound to the protein surface.³³ This second calcium binding site could be important for binding Pu⁴⁺-NTA. GRP78 is known to bind divalent metal ions such as Pb²⁺, Cu²⁺, Zn²⁺, and Ni²⁺.³⁴ It also binds trivalent lanthanide cations (La³⁺, Gd³⁺) in the calcium binding site, stimulating the production of reactive oxygen species (ROS) by altering Ca²⁺ homeostasis.³⁵ The previously reported affinity of GRP78 for the lanthanides La³⁺ and Gd³⁺ is consistent with its observed affinity for the Ho³⁺-NTA IMAC column. GRP78 is highly conserved in mammals and over-expressed in endoplasmic reticulum lumen. It is highly induced in response to glucose regulated stress and provides resistance to apoptosis.³⁶ Over expression of GRP78 in cancer cells and its anti-apoptotic function indicates possible involvement of this protein in cancer development, such as colorectal carcinogenesis.³⁷ Progression of melanoma and malignant potential of astrocytomas were also found to be associated with the

GRP78 status.^{38, 39} More recently, GRP78 was found to be one of the regulators of the host vasculature within the tumor microenvironment.⁴⁰

The other two plutonium-binding proteins that were previously reported to bind uranyl (nucleoside diphosphate kinase B spot 2 and predicted actin, gamma 1 propeptide-like isoform spot 3) are not well characterized with respect to native metal binding. A 17 kDa nucleoside diphosphate kinase B is known to catalyze phosphate transfer and it also binds magnesium.⁴¹ The nucleoside diphosphate kinase B is involved in cell proliferation and differentiation. Several studies have suggested the strong correlation of this protein in tumor metastasis.^{42, 43} NDK B has even been proposed as a biomarker for lung cancer;⁴⁴ and as a cell surface marker for leukemia cells.^{45, 46} The uncharacterized 41.79 kDa predicted actin, gamma 1 propeptide-like protein from rat is a cytoplasmic protein found in non-muscle cells. It has not been well studied for metal binding but actin is well known to bind Ca^{2+} and Mg^{2+} ions with micromolar affinity.⁴⁷ Moreover, the cytoplasmic G actin concentration is critical for stimulus response cell extension;⁴⁸ this function has a role in cell motility and may therefore be of importance in cancer progression too.

The 14.7 kDa protein identified as rat galectin-1 (spot 4, Figure 1) is a member of a family of carbohydrate binding proteins.⁴⁹ It is highly expressed in skeletal muscles and is found in extracellular matrices. Although the metal binding properties of galectin-1 are unknown, galectin-1 induces an increase in the intra-cytoplasmic free calcium ion concentration.⁵⁰ Galectin-1 is associated with negative regulation of cell-substrate adhesion and the cellular response to glucose stimulus.^{51, 52} The over expression of this protein in glucose regulated stress provides resistance to apoptosis. This protein is upregulated in a great variety of human cancers such as lymphoid, prostate, lung, breast, pancreas, skin, urinary, kidney, upper digestive, thyroid, stomach, colon, ovary, and uterine cervix cancers.⁵³ In addition, galectin-1 has been found to play a role in metastasis of oral squamous cell carcinoma.⁵⁴

Another plutonium-binding protein we identified is rCG57843 isoform CRA_a (spot 5, Figure 1); which has a 100% sequence identity with rat M2 pyruvate kinase (GI:206205). M2 pyruvate kinase catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule ATP and a pyruvate molecule. It binds Mg^{2+} or K^{+} as a cofactor. This protein isoform plays a critical role in tumor characteristic aerobic glycolysis.^{55, 56}

Similarly other two proteins: the complement component 1 Q subcomponent-binding protein (gC1q-R), and 20 kDa alpha crystallin proteins, which were identified with only two matching peptide sequences, also show some metal binding properties as well as important anti-apoptotic function. The protein gC1q-R binds C1q (complement component 1 q subcomponent) and has an important regulatory role in infection and inflammation.^{57, 58} It is also involved in intracellular adhesion.⁵⁹ gC1q-R from spot 6 is a well isolated protein in the 2D gel that always appeared after separation with the Pu^{4+} -NTA column but did not appear in 2-D gels of proteins isolated from cell lysates by the Fe^{3+} -NTA or Ln^{3+} -NTA columns. This protein is of particular interest for detailed plutonium-binding studies. It has an aspartic and glutamic acid rich surface with a theoretical pI of 4.77 for the full length protein. The crystal structure of the apo human mitochondrial gC1q-R (p32) trimer has been solved to 2.25 Å resolution.⁶⁰ In the crystal structure three p32 molecules fold into a doughnut-shape with a strongly asymmetric surface charge distribution. One side of the protein is more negatively charged than the other, and the negatively charged residues are primarily concentrated in the loops that connect β -strands. The negative surface is predicted to serve as divalent cation storage site; especially for calcium ions. A specific calcium binding site was not characterized in the crystal structure (PDB ID: 1P32),⁶⁰ but a shallow pocket formed by E89, E93, L231, D231, and Y268 on the negatively charged side of doughnut

between two p32 molecules may serve to specifically bind calcium or, in our case, plutonium. The absence of this protein spot from lanthanide or iron binding systems suggests that non-specific binding to the Pu⁴⁺-NTA column is not important.

Alpha crystallin (spot 7, Figure 1), a member of small heat shock protein family, is known to bind metal ions such as copper, zinc, and calcium,⁶¹ but its lanthanide or actinide binding properties have not been studied yet. However, the related protein beta crystallin is known to bind terbium with an affinity higher than calcium⁶² indicating the possible involvement of crystallins in actinide and lanthanide binding. Alpha crystallins act as negative regulators of apoptosis by inhibiting proteolytic inhibition of caspase-3.^{63, 64} This suggests an anti-apoptotic function for crystallins.

Most of the possible plutonium-binding proteins identified in this study show metal binding properties for Mg²⁺, Ca²⁺, or transition metals. In the proteins with known crystal structures, Ca²⁺ or Mg²⁺ ions are coordinated with oxygen atoms from the phosphate of ADP/ATP in the active site, or aspartate or glutamate residues with 3–5 coordinating water molecules. Similar to calcium, lanthanides and actinides also prefer hard donor ligands and coordination environments with numerous donor atoms (coordination numbers of 8 or 9 are typical). Many calcium-binding proteins are known to bind lanthanides effectively,⁶⁵ and the calcium-binding protein, calmodulin, is also reported to bind Pu³⁺ in the calcium binding sites⁶⁶. Therefore, the proteins identified in this study are reasonable candidates for plutonium binding *in vivo*. It is also interesting to note that most of the proteins isolated from the Pu-NTA affinity column in our study are normally found in the cytoplasm. Our previous X-ray fluorescence imaging experiments of PC12 cells incubated in the presence of plutonium showed the cytoplasm to be the primary early site of plutonium deposition in this cell line.¹³ Prior attempts to study the sub-cellular distribution of plutonium by fractionation of the organelles also found small amounts of plutonium in the mitochondrial and nuclear fraction⁶⁷. Thus it is not surprising to find plutonium-binding proteins from other parts of the cell, such as the nuclear proteins galactin-1 and nucleoside diphosphate kinase B and the mitochondrial protein gClq-R.

The known affinity of plutonium for iron(III)-binding biomolecules such as the metalloproteins transferrin and ferritin or bacterial siderophores suggests that plutonium will partially follow iron(III) biochemical pathways. This has been important in developing the current understanding of the *in vivo* behavior of plutonium, and current biokinetic models of plutonium's behavior in the body are mainly based on this idea⁶⁸ even though cellular plutonium transport systems are not understood in any detail. The results of our proteomic analysis present a different picture of potential intracellular plutonium binding proteins. None of the proteins identified are known to function as iron(III) binders, which suggests that proteins without an affinity for ferric ions might also be important in understanding plutonium's chemistry in the cell. For another actinide element, uranium, metalloproteomic approaches have also identified several uranyl interacting proteins in human serum and kidney-2 cell extracts.^{22, 69} Some of those proteins also show divalent metal (Ca²⁺, Mg²⁺, Zn²⁺) binding properties while others have no known relationship to metal homeostasis. The proteins identified in this work likely do not have as high affinity for plutonium as transferrin or bacterial siderophores, which are extracellular transport agents. However, intracellular proteins with comparatively lower plutonium affinities could still be important in intracellular trafficking.

Metalloproteins play critical structural, catalytic, and regulatory roles in cells. While cells have not evolved in presence of plutonium, plutonium still interacts with cellular proteins, and causes cellular damage through irradiation and also possibly by chemical means. By themselves, our proteomic studies are unable to unravel questions about the nature of

plutonium's interactions with individual proteins, for example if plutonium binds in specific binding sites, and how many plutonium ions are associated with each protein, yet these proteins present interesting targets for further study. More importantly involvement of these proteins in anti-apoptotic processes and cell substrate adhesion suggests the possible biochemical roles in cancer development. Thus detailed study of the interaction of these proteins with plutonium will help to understand plutonium uptake, distribution, and plutonium mediated carcinogenic effects.

MetaCore™ Analysis

All the identified proteins with interact with plutonium. Interestingly, they all are involved in GO processes as well. After analyzing the functions and GO processes for individual Pu binding proteins, we focused on cellular processes and pathologies associated with these proteins as a group. MetaCore “shortest path” pathway analysis was performed. A list of all of the proteins derived from the analysis, their most frequent subcellular locations, and the number of interactions (referred to as “edges” by MetaCore) involved in the pathway presented is given in Supplemental Table S1. We found that together GRP78, actin gamma 1, NDPK B, and galectin 1 regulate the GO processes listed in Table 2. The percentage of proteins from the network included in the analysis is given as well, together with probability of the GO process indicated.

The schematic of the “shortest distance” pathway map constructed by GeneGO is presented in Figure 2. We added Transferrin receptors 1 and 2 to the pathway build-up, because of the established association between transferrin and Pu.^{14, 15} In this figure, Pu interacting proteins are presented together with other proteins with which they have a relationship curated in GeneGO. Diseases associated with the collection of proteins involved in the “shortest distance” pathway (shown in Figure 2) built to encompass the Pu binding proteins from this study and based on canonic pathways available in GeneGO database are shown in Table 3. Based on this pathway as a whole, GeneGO analysis suggests a list of twelve most probable pathological processes, 10 of which are neoplastic disorders (Table 3).

CONCLUSIONS

This study represents the first report of metalloproteomic analysis of potential plutonium binding proteins in mammalian cells. Our results show that in addition to the iron binding proteins transferrin and ferritin, which have long been known to bind plutonium, there are other proteins that interact with plutonium and could be involved in intracellular Pu trafficking and sequestration in mammalian cells. Proteins identified from the Pu-NTA IMAC have anti-apoptosis roles and most are known or predicted to bind other metal ions. The interactions of plutonium with proteins that have anti-apoptotic functions suggest that plutonium may selectively stimulate these proteins for cancer development in a new way, which remains to be understood and requires thorough investigation. Even if plutonium does not induce cancer directly through chemical means, might the intracellular biochemical interactions of plutonium predispose cells for radiation-induced neoplasia? More experiments are required to understand the specific or non-specific binding of plutonium with these proteins, the role of these individual proteins for plutonium trafficking inside the mammalian cells, and the possible carcinogenic effects of plutonium's interactions with these proteins. Expression, purification, and full characterization of the identified proteins are underway to understand how plutonium interacts with these proteins and the possible roles of these proteins in plutonium uptake and distribution within the cells. Understanding the plutonium-binding properties of these proteins will help researchers understand the mechanisms of plutonium toxicity, knowledge that can be translated to more effective therapeutic agents to protect radiation workers and civilians from plutonium poisoning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Silver stained image (A) of gel from 2D SDS-PAGE of proteins isolated from Pu^{4+} -NTA IMAC. The horizontal line indicates the pI from 3–10 and the vertical line denotes the molecular mass. Proteins in spots 1 and 4 were identified from a different set of gels. Only the identified proteins are labeled as spots 1 to 7. Figure B represents the selected part of the same gel after adjusting the exposure (brightness and contrast). Spot 1 is missing in figure B.

Figure 2.

A “shortest distance pathway” connecting the proteins identified to be involved in Pu binding, directly or indirectly (via transferrin). The proteins identified by 2DE and mass spectrometry with at least 4 peptides are shown at the bottom of the figure (See also Table 1). Every arrow represents a curated relationship between proteins connected; red arrows are inhibitions, green arrows activations and gray arrows unspecified (or more complex) relationships. One “upstream” and one “downstream” relationship from GRP78 are highlighted by light blue as examples.

Table 1

Plutonium binding proteins identified by LC-MS/MS analysis

Spot No	Proteins	Accession number	Number of peptides	Sequence coverage %	Mol. Wt. kDa	Protein functions (metal binding and selected GO processes for single proteins)
1	78 kDa glucose-regulated protein precursor	GI:25742763	8	14	72.34	Calcium dependent molecular chaperone induced to assist protein folding, binds Ca^{2+} ATP or ADP GO process: anti-apoptotic, negative regulation of apoptosis
2	Nucleoside diphosphate kinase B.	GI:55926145	4	15	45.24	Catalyzes phosphate transfer, binds Mg^{2+} GO process: negative regulation of apoptosis
3	PREDICTED: actin, gamma 1 propeptide-like	GI:293342999	4	11	41.79	Cell motility GO process: response to calcium ion
4	Rat galectin-1	GI:9845261	6	49	14.74	Carbohydrate binding GO process: cellular response to glucose stimulus, negative regulation to cell-substrate adhesion
5	rCG57843, isoform CRA_a (M2 pyruvate kinase GI: 206205)	GI:149041863	4	12	57.7	Involved in glycolysis and ATP production GO process: glycolysis
6	complement component 1 Q subcomponent-binding protein (gC1q-R protein)	GI:48675371	2	10	30.99	Ca^{2+} binding and storage GO process: involved in intercellular adhesion and in the clearance of apoptotic cells, cell adhesion
7	alpha crystallin B chain	GI:16905067	2	12	20.05	Binds Ca^{2+} , Zn^{2+} , Cu^{2+} GO process: anti-apoptosis

Table 2

GO Processes for a “shortest distance” network including Pu binding proteins GRP78, actin gamma1, NDPK B and galectin 1.

GO Processes for a multi-protein network	% genes included in the pathway	p-Value for the GO process for the fraction of genes involved
regulation of apoptosis	58.33	2.287e-11
regulation of programmed cell death	58.33	2.610e-11
developmental process	87.50	2.837e-11
response to organic substance	66.67	3.226e-11
regulation of cell death	58.33	3.675e-11
response to chemical stimulus	79.17	9.417e-11
response to inorganic substance	41.67	1.227e-10
macromolecule metabolic process	91.67	5.144e-10
regulation of caspase activity	29.17	7.030e-10
system development	75.00	1.099e-09
response to stimulus	95.83	2.509e-09
response to metal ion	33.33	3.788e-09

Table 3

Diseases associated with the collection of proteins involved in the “shortest distance” pathway (shown in Figure 2).

GO Diseases (listed in order of probability)	% of all genes listed	p-Value for gene-disease association
Neoplasms, Connective and Soft Tissue	52.38	1.979e-15
Sarcoma	47.62	3.116e-15
Neoplasms, Germ Cell and Embryonal	76.19	3.305e-15
Neuroectodermal Tumors	73.81	1.575e-14
Neoplasms, Nerve Tissue	73.81	2.519e-14
Carcinoma	83.33	6.810e-14
Signs and Symptoms, Respiratory	42.86	9.378e-14
Adenocarcinoma	73.81	3.720e-13
Hematologic Diseases	61.90	4.504e-13
Neoplasms, Neuroepithelial	64.29	4.799e-13
Neuroblastoma	38.10	9.930e-13
Neuroectodermal Tumors, Primitive, Peripheral	38.10	1.454e-12