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Large scale phosphoproteome analysis of LNCaP human prostate cancer cells

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Abstract

Prostate cancer is the most frequently diagnosed cancer among men in the western world. The androgen receptor, a phosphoprotein, is suspected to be involved in all stages of the prostate cancer. Androgen receptor activity can be modulated by various kinases such as PKA, MAPK, AKT, and Src. Phosphorylation is an important post-translational modification and serves as a molecular on/off switch to regulate signaling. Disruptions of cellular phosphorylation are associated with various diseases such as cancer and kinases provide important drug targets. Here we present an analysis of the phosphoproteome in LNCaP human prostate cancer cells. The analytical strategy employed used proteomics based methodologies with a combination of detergent and chaotropic reagent during trypsin digestion followed by titanium dioxide enrichment of phosphopeptides. Over the course of multiple analyses by mass spectrometry we identified a total of 746 phosphorylation sites in 540 phosphopeptides corresponding to 116 phosphoproteins, of which 56 have not been previously reported. Phosphoproteins identified included transcription factors, co-regulators of the androgen receptor, and cancer-related proteins that include β -catenin, USP10, and histone deacetylase-2. The information of signaling pathways, motifs of phosphorylated peptides, biological processes, molecular functions, cellular components, and protein interactions from the identified phosphoproteins established a map of phosphoproteome and signaling pathways in LNCaP cells.

Introduction

Prostate cancer is the most common cancer in men and the most frequent cancer-related death after lung cancer in Europe and North America. A comprehensive understanding of the pathways and molecules that affect the heterogeneous progression of prostate cancer to an advanced terminal stage is required to identify mechanisms and therapeutic targets to improve the clinical management of the disease. Important pathways suspected to be involved in the progression of prostate cancer include the androgen receptor (AR) and various kinases such as PKA, MAPK, AKT, erbB2, and Src^{1–7}.

Protein phosphorylation is the most widespread post-translational modification (PTM) in nature and occurs on at least one third of all proteins in mammalian cells⁸. Phosphorylation of proteins by a series of kinases with specific activities in different systems can regulate protein function, turnover, cellular localization and various biological processes such as signaling pathways by triggering a conformational change, subcellular location, generating binding sites for an interacting partners or altering its stability. Phosphorylation of nuclear receptors such as the AR and their coactivators alters their subsequent transcriptional

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activities. Changes in phosphorylation of AR may directly alter protein-protein interactions or indirectly alter interactions through changes in other PTMs, such as sumoylation and acetylation, or lead to changes in degradation, expression, and cellular localization of essential proteins. The disruption of phosphorylation events in a cell or tissue is associated with many diseases including cancers such as prostate cancer.

Development of global and quantitative methods for elucidating phosphorylation events is essential for biochemical analysis of cellular events that may be involved. Mass spectrometry (MS) based analysis is a powerful technology for proteomics and a method of choice for phosphorylation owing to its high sensitivity and ability to identify phosphorylation sites by MS/MS sequencing^{9, 10}. Functional proteomics techniques coupled with MS have contributed to prostate cancer study through revealing the molecular mechanisms and biological processes by analyzing protein-protein, DNA or RNA interactions as well as PTMs. Examples include discovery of potential biomarkers for prostate cancer using SELDI¹¹ and SILAC¹², and identification of proteins that interact with AR by MudPIT¹³. Phosphorylation of a number of individual molecules have been identified and shown to have biological effect on important signaling pathways in prostate cancer^{14–19}. To date, there are two phosphoproteome analysis using MS-based approach to identify phosphoproteins in prostate cancer cells^{20, 21}. Here we identify phosphoproteins in LNCaP human prostate cancer cells with a combination of detergent and chaotropic reagent use during trypsin digestion to provide a global view of regulation of signaling pathways by phosphorylation and produce a reference for the phosphoproteome of a model of prostate cancer.

Results

To identify the phosphoproteome in a model of prostate cancer, we used the well-characterized LNCaP human prostate cancer cells. These cells can be maintained *in vitro* or grown *in vivo* as xenografts. LNCaP cells originated from a lymph node metastasis and express the AR and prostate-specific antigen (PSA), which is the clinical biomarker for prostate cancer. LNCaP cells are responsive to androgens and progress to the castration recurrent stage thereby mimicking several important aspects of the disease. The phosphoproteome strategy (Fig. 1) employed here successfully identified 540 phosphopeptides. Some of these phosphoproteins are known as cancer-related proteins and AR-interacting molecules. Creation of a map of phosphoproteome in LNCaP cells may aid in elucidating signaling pathways involved in molecular functions, biological processes, and cellular components.

Effect of sodium deoxycholate and urea on trypsin digestion for phosphopeptide preparation

The success of large-scale phosphoproteome analysis is dependent on efficient methods to extract and enrich for phosphopeptides from complex samples with minimal contamination by non-phosphorylated peptides. Chromatographic resins such as immobilized metal affinity chromatography (IMAC) and TiO₂ can be applied for enrichment of phosphopeptides from a mixture of peptides. Different protocols for purification of phosphopeptides may result in varying efficiencies. Here we used TiO₂ which is more stable than IMAC in detergents, salts and buffers that are frequently used in biological experiments²². NP-40 in Tris-HCl lysis buffer with proteases and phosphatase inhibitors was used based upon its use in successful analysis of the phosphoproteome of Jurkat cells¹⁰.

Protein digestion is critical step as it produces peptides from intact proteins for MS analysis. Chaotropes and some detergents can be added to improve denaturation of proteins for MS-based analyses. Enzyme digestion of proteins in solution with MS-compatible reagents

improves the protein identification ratio by generating more diverse mixture of peptides. Conditions and various MS-compatible reagents have been reported and compared for enzyme digestion^{23, 24}, but require optimization for maximum identification of proteins in a specific experiment.

Here we used both NaDOC and urea during enzyme digestion to increase the efficiency of identification of phosphoproteins which previously have shown to improve tryptic digestion and identification of proteolytically resistant proteins compared to standard trypsin digestion^{23, 24}

No significant difference in phosphopeptide identification between two surfactants was observed. There were 125 and 127 unique phosphopeptides corresponding to 90 and 89 phosphoproteins that were identified using NaDOC and urea containing trypsin digestion buffer, respectively. Of these, 73 of 125 (58.4%), and 71 phosphopeptides of 127 identified total phosphopeptides (55.9%) in NaDOC and urea, respectively, were completely digested thereby demonstrating a possibly slightly better efficiency of NaDOC in trypsin digestion. Interestingly, more proteins with positive GRAVY values and with molecular weights higher than 200 were observed in the presence of NaDOC (Table 1). However, there was no significant difference in the average pI obtained using these reagents.

Identification of phosphoproteins in LNCaP cells by MS

Enriched phosphopeptides were analyzed by high accuracy MS analysis and the combination of two surfactants: detergent (sodium deoxycholate); and chaotropic reagent (urea) during trypsin digestion. Proteins identified for each group, including peptides identified per protein, protein ID, protein name, protein sequences, charge state, product ion mass were provided in Supplementary Table 1. A total of 746 phosphorylation sites in 540 phosphopeptides were identified in LNCaP cells by phosphopeptide-specific approach using TiO. These 540 phosphopeptides corresponded to 116 unique phosphoproteins of which 56 phosphoproteins have not been previous reported in the phosphoproteome of LNCaP cells (Supplementary Table 2). These data are consistent with previous reports of proliferating LNCaP cells where the phosphor-levels seem relatively low with identification of 81 and 296 phosphoproteins^{20, 21}. Proliferating cells are reported to have a relative abundance of 90% phosphoserine (pS), 10% phosphothreonine (pT), and 0.05% phosphotyrosine (pY)²⁵. Here the distribution of individually identified phosphorylation sites suggested a similar distribution of pS, pT and pY sites that was 88%, 12% and <1%, respectively.

The majority of identified proteins contained one unique peptide hit (Table 2). Most phosphopeptides were singly phosphorylated (72%), but doubly (26%) and triply (2%) phosphorylated peptides were also identified.

Classifications of phosphoproteome into biological process, molecular function and cellular component

All identified proteins were categorized according to biological process, molecular function, and cellular component by Gene Ontology (GO) analysis using Discoveryspace²⁶. The total number of proteins identified by LC-MS/MS analysis was found to represent various biological processes (Fig. 2A). The top five biological process categories of phosphoproteins identified in LNCaP cells were: 1) signal transduction (12%); 2) regulation of transcription (12%); 3) mRNA processing (8%); 4) cell differentiation (8%); and 5) RNA splicing (7%).

The identified proteome in LNCaP cells was also classified into molecular functions (Fig. 2B) and revealed that 28% of phosphoproteins were classified to have the molecular function of protein binding, followed by 22% that were involved in nucleic acid binding, 14% with nucleotide binding, and 7% with hydrolase activity.

The cellular localization of phosphoproteins were determined to be localized in cytoplasm (46%) followed by plasma membrane (10%) in the presence of NaDOC or urea. However trypsin digestion with NaDOC led to identification of more membrane proteins than urea (21% and 18% of plasma membrane, ER membrane, and membrane fraction in NaDOC and urea, respectively) (Fig. 2C and 2D)

Signaling pathways and predicted motifs of identified phosphopeptides

Phosphorylation plays an important role in the control of signaling pathways that are dependent on the activities of protein kinases. Signaling pathways involving the identified proteins were categorized and the phosphorylation motifs predicted for the identification of targets of phosphorylation using PANTHER (<http://www.pantherdb.org/>) which uses a large collection of 6,683 protein families that cover 90% of mammalian protein-coding genes²⁷ and categorizes identified genes into signaling pathways. Proteins involving Wnt signaling pathway represented the largest group of 35 different signaling pathways. Proteins involved in cadherin signaling pathway represented the second largest group. Other pathways included Rho GTPase, heterotrimeric G-protein signaling, Gi alpha and Gs alpha mediated pathway, and Alzheimer disease-presenilin pathway (Fig. 3).

To identify potential common phosphorylation motifs, PHOSIDA was employed. This is an algorithm/ database designed to extract motifs from large sets of naturally occurring phosphorylation sites. A query of our dataset for phosphopeptides was predicted to have 20 different motifs and the majority of identified phosphopeptides was demonstrated to have motifs for casein kinase (CK) 1 and 2 followed by GSK3 (Table 3).

Androgen receptor and its interacting proteins

AR is suspected to be important in all stages of prostate cancer. Although AR is a phosphoprotein, it was not detected here with either approach. The inability to detect phosphoAR is possibly due to low expression level and/or the requirement to use a combination of trypsin and Glu-C digestion to obtain optimally-sized cleavage products for MS analysis. Approximately 170 proteins that are involved in a variety of processes have been reported to interact with the AR²⁸. Here we identified 3 proteins which are known to interact with AR as a phosphorylated form (Table 4). These proteins were histone deacetylase 2 (HDAC2) (Fig. 4A), ubiquitin carboxyl-terminal hydrolase 10 (USP10) (Fig. 4B) and β -catenin (CTNNB1) (Fig. 4C). Data from MS analysis including phosphorylation sites identified by Mascot are shown in Table 5. These sites correspond to casein kinase-2 motif (S/T-X-X-E) in serine 8 and 10 of HDAC2 and in serine 16 of USP10 as well as PKA motif (R-X-S/T) in serine 3 of CTNNB1.

Mapping a network of identified cancer-related molecules and molecules involved in prostate cancer

The association of the cancer-related molecules among identified phosphoproteins and the relationships to prostate cancer was mapped using Ingenuity Pathways Analysis (IPA) (Fig. 5). A total of 61 molecules were observed to be involved in the networks related with cancer function directly or indirectly. Molecules identified as phosphorylated forms that play important roles in prostate cancer were splicing factor 1 (SF1), regulator of cohesion maintenance homolog B (PDS5B), nucleolin (NCL), β -catenin (CTNNB1), histone deacetylase 2 (HDAC2), and minichromosome maintenance complex component 2 (MCM2). All phosphorylation sites of these proteins were previously identified in a large scale phosphoproteome analysis in HeLa cells and HEK293 cells^{29–31}, but the role of phosphorylation and phosphorylation-induced molecular actions have not been reported. Dysregulation of these molecules may give rise to initiation and progression of cancer and provide potential targets for therapeutic intervention.

Discussion

Analysis of the phosphoproteome of cells can provide a detailed view of signaling pathways and elucidate potential targets for rational drug development. The phosphoproteome has been analyzed in numerous cancer cell lines including human leukemic monocytic lymphoma U937 cells³², human colon adenocarcinoma HT-29 cells³³, B lymphoma WEHI-231 cells³⁴, Hela cervical cancer cells³⁵ and lung adenoma carcinoma A549 cells³⁶.

In an effort to identify phosphoproteins and signaling pathways in prostate cancer, we employed the MS analysis combined with highly selective TiO₂ phosphopeptide enrichment method using LNCaP cells as a model for prostate cancer. Both NaDOC and urea were used during enzyme digestion to maximize the identification of phosphoproteins. It is necessary to consider the compatibility of reagent with MS as it can interfere with analysis by obscuring and/or suppressing the signal, formation of adducts, and shift the charge states. Extensive work has been done to evaluate the compatibility of a variety of surfactants on MS³⁷ and urea and NaDOC are generally accepted. Urea is a commonly used chaotropic reagent to denature proteins as it is easily removed by reverse-phase liquid chromatography. NaDOC is a detergent that is compatible with MS and can be used at high concentrations to improve trypsin digestion and identification of proteins^{38, 39}. The combination of two different surfactants for trypsin digestion showed slightly increased identification efficiency of phosphoproteins. Phosphoproteome analysis with NaDOC containing enzyme digestion demonstrated slightly increased identification of phosphopeptides and hydrophobic proteins, as well as improved efficiency of trypsin digestion. Importantly here we identified a total of 116 phosphoproteins of which 56 had not been previously reported in LNCaP cells. Of 116 phosphoproteins, 28 and 27 proteins uniquely identified by urea and NaDOC buffer, respectively. Technical steps to improve analysis of phosphoproteome become paramount in cell lines or samples where the phosphor-levels are relatively low during regular growth of cells or in the absence of stimulation with growth factors.

Analysis of the phosphorylation motifs on the identified proteins yielded an abundance of sites for the serine/threonine specific protein kinase family, CK1 and CK2, as well as glycogen synthase kinase 3 (GSK-3). CK1 is ubiquitously expressed in eukaryotic organisms and mediates the phosphorylation of many substrates that are involved in various cellular processes including cell differentiation, proliferation, membrane transport, and oncogenesis. CK2 is implicated in cell growth and can affect prostate cancer development^{40, 41}. Inhibition of CK2 activity decreases AR protein and AR-dependent transcription⁴². GSK-3 is widely expressed in mammalian tissues and phosphorylates members of the steroid receptor family^{43–45}, including AR, but its effects on AR transcriptional activity remain controversial^{43, 46–48}. Phosphoproteins identified with motifs for GSK3 was MAP1B⁴⁹ and for CK2 were SSB⁵⁰, IGF2R⁵¹ and YBX1⁵².

There is increasing evidence that hormonal progression of prostate cancer is mediated through aberrant activity of the AR by overexpression of the AR and/or altered interactions with AR coregulators such as coactivators and corepressors. Here we identified phosphorylated USP10, HDAC2, and β -catenin, which interact with AR. The phosphorylation sites identified are novel hence no functional studies have been reported that characterize these sites.

HDACs influence differentiation, and proliferation of cancer cells. HDAC2 is suggested to be an independent prognostic marker for prostate cancer patients and is associated with biochemical failure and recurrence⁵³. Phosphorylation of HDAC2 by CK2 on ser394 and potentially ser411, ser422, and ser424 is known to promote enzymatic activity and regulates

complex formation, but has no effect on transcriptional repression⁵⁴. We identified ser422 and ser424 phosphorylation sites of HDAC2 that lie within CK2 recognition sequences.

β -catenin is a multifunctional molecule that plays important roles in cell-cell adhesion and Wnt signal transduction. In the absence of Wnt signaling, protein levels of β -catenin are regulated by phosphorylation on its NH2-terminal region by GSK3 which leads to its ubiquitination and degradation. Wnt signaling inhibits this process to cause accumulation of β -catenin such that it can translocate to the nucleus and modify the activity of Tcf/Lef transcription factors⁵⁵. β -catenin is an important coactivator of the AR and thought to be a major player in prostate cancer⁵⁶. Here we identified phosphorylated β -catenin which was consistent with a previous study using this cell line²⁰.

USP10 is an ubiquitin-specific protease that we identified as a phosphoprotein in LNCaP cells. The biological role of USP10 is not fully understood, but it is known to interact with DNA-bound AR complexes⁵⁷. USP10 has been detected as a phosphoprotein in Hela cells^{35, 58}, HEK293 cells⁵⁹, human embryonic kidney 293T cells⁶⁰, and consistent with our data, also in LNCaP cells²⁰. Physical interaction between USP10 and G3BP, a RasGAP interacting protein overexpressed in tumors, was shown by a two-hybrid screen to suggest a link between the ubiquitination pathway and Ras-mediated signaling⁶¹.

More than half of the identified phosphoproteins were mapped to relate with cancer function. These phosphoproteins include, splicing factor 1 (SF1)⁶², sister chromatid cohesion protein PDS5 homolog B (PDS5B)^{63, 64}, nucleolin (NCL)⁶⁵, β -catenin^{66, 67}, histone deacetylase 2 (HDAC2)⁶⁸, and DNA replication licensing factor MCM2 (MCM2)⁶⁹ and were linked into prostate cancer disease using IPA analysis. Phosphorylation of MCM2 is involved in the initiation of DNA replication in Hela cells⁷⁰. Elucidation of the role of phosphorylation of these molecules may provide insight into the molecular mechanisms underlying prostate cancer.

Materials and Methods

Cell Culture

LNCaP human prostate cancer cells (L.W.K. Chung, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center) were maintained in phenol red-free RPMI 1640 culture medium (Stem Cell Technology, Vancouver, BC) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100units/mL penicillin and 100ug/uL streptomycin (Stem cell technology). All other chemicals were from Sigma-Aldrich (Oakville, ON) unless stated.

Sample Preparation

LNCaP cells which have approximately 36 hours-doubling time were harvested in log-phase growth (approximately 80% confluent). Protein lysates were prepared by tissue homogenizing using 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM NaF, 5 mM Na₃VO₄, 10 mM beta-glycerophosphate, and complete-mini EDTA free protease inhibitor cocktail tablets (Roche Applied Science, Laval, Quebec). Nucleic acids were degraded using benzonase (Novagen, Mississauga, ON) and lysates were clarified by centrifugation at 20,000 $\times$ g for 10 min at 4°C. Protein concentrations were determined using a bicinchoninic acid protein assay (BioRad, Hercules, CA).

Trypsin digestion

Proteins from two aliquots of LNCaP lysate were precipitated for 16 hours at -20°C with 9 volumes of ice-cold acetone. Protein pellets were collected by centrifugation at 20,000 $\times$ g

for 30 min at 4°C. Proteins were resolubilized using either 8 M urea or 1% sodium deoxycholate (NaDOC). Samples were reduced with dithiothreitol and alkylated with iodoacetamide prior to dilution with 50 mM ammonium bicarbonate (pH 8.0) to reduce concentration of urea to level compatible with trypsin activity (<1 M). Trypsin (sequencing grade) (Promega, Madison, WI, USA) was added at a 50:1 substrate:enzyme ratio and digestion was conducted for 16 hours at 37°C. Trypsin digestion was stopped with the addition of formic acid to a final concentration of 1%. NaDOC is insoluble below pH 2.0 and was removed by centrifugation for 5 min at 20,000 × g at room temperature.

Enrichment of phosphopeptides

Phosphorylation is often substoichiometric thereby necessitating an affinity enrichment procedure prior to analysis and detection by MS. Here we use titanium dioxide (TiO₂) affinity method that is highly selective for phosphorylated peptides⁷¹. Prior to TiO₂ binding, digested peptides were desalted using reversed phase solid phase extraction with Waters Oasis HLB cartridges. Peptides were eluted from the HLB columns using 500 µL of TiO₂ binding buffer (80% acetonitrile, 0.1% trifluoroacetic acid (TFA), 300 mg/mL dihydroxybenzoic acid (DHB))⁷². TiO₂ binding buffer was added to TiO₂ beads (GL Sciences, Japan) to create 50% slurry. TiO₂ microcolumns and phosphopeptide enrichment was conducted as described⁷¹.

Briefly, TiO₂ bead slurry was added to each desalted LNCaP digest and incubation was conducted at room temperature for 30 min. TiO₂ bead slurries were transferred to individual fitted microcolumns. TiO₂ beads were washed with TiO₂ binding buffer, followed by wash buffer (80% acetonitrile, 0.1% TFA). Bound peptides were eluted with 20 µL of ammonium hydroxide and eluted samples were acidified with an equal volume of 10% formic acid.

Mass spectrometry analysis

Analysis of peptides was conducted using an LC Packings Famos Autosampler with an LC Packings Ultimate nanoflow HPLC coupled to a nano-electrospray ionization source on a QSTAR Pulsar I (Applied Biosystems, Streetsville, Ontario). The HPLC was operated with a desalting column (0.3 × 5 mm) HPLC plumbing configuration to protect the analytical column (Magic C₁₈ resin, 150 mm × 75 µm inner diameter). Samples were loaded onto the desalting column at 50 µL/min with 100% solvent A (2% v/v acetonitrile, 0.1% formic acid) for 5 min. Upon switching the desalting column in-line with the analytical column, a 300 nL/min gradient was used as follows: 60 min linear gradient from 0–20% solvent B (98% v/v acetonitrile, 0.1% formic acid); 30 min linear gradient from 20–40% solvent B; 12 min linear gradient from 40–80% solvent B.

Tandem MS spectra were acquired in a data dependent fashion selecting the top 2 most intense eluting ions in the 400–1200 m/z range with a 2+ to 5+ charge state. Following MS/MS analysis, each precursor ion was excluded from selection for further MS/MS analysis for 180 s. Raw MS/MS data was centroided, deisotoped and converted to peak lists using mascot.dll (version 1.6b23, Matrix Science, London, UK) for Analyst QS 1.1. Peak lists were queried against the Human IPI database version 3.43 (72,350 sequences) using Mascot 2.2 (Matrix Science). Search parameters included trypsin enzyme specificity with 1 missed cleavage permitted, and 0.3 Da and 0.1 Da precursor and fragment ion mass error, respectively. Carbamidomethylation of Cys was allowed for fixed modifications and deamidation of Gln and Asn, oxidation of Met, and phosphorylation of Ser, Thr and Tyr were allowed as variable modifications.

Computational analysis

All identified phosphoproteins were categorized according to signaling pathways, biological process, cellular component and molecular function using PANTHER (<http://www.pantherdb.org/>) (version 4.01)²⁷ and Discoveryspace (version 2.1)²⁶ for the identification of targets of reversible phosphorylation. PHOSIDA (<http://www.phosida.com>) was used to predict phosphorylation motifs involved. PHOSIDA is designed to extract motifs from large sets of naturally occurring phosphorylation sites matching kinase motifs, predicted secondary structures, conservation patterns, and its dynamic regulation upon stimulus⁷³. bioDBnet (<http://biodbnet.abcc.ncifcrf.gov/db/db2db.php>)⁷⁴ was used to convert UniProt ID to IPI to compare proteins identified in our study with in other two studies^{20, 21}.

Ingenuity Pathways Analysis (IPA) (version 6.3) aids in identifying interactions and functions of gene products using information based on the literatures. IPA was used to map the connection of identified and cancer related gene products as well as their involvements of signaling pathways (<http://www.ingenuity.com/>).

Molecular weights, theoretical isoelectric point (pI) and Grand Average of Hydropathy (GRAVY) value were calculated using ProtParam (<http://www.expasy.org/tools/protparam.html>) for each protein. The GRAVY value for a peptide or protein was calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence and shows the hydrophobicity of proteins⁷⁵.

Conclusion

In conclusion, the application of detergent and chaotropes with enzyme digestion followed by a phosphopeptide enrichment strategy yielded identification of 116 phosphopeptides, of which 56 have not been reported previously in LNCaP human prostate cancer cells. Identification of the phosphoproteome reveals valuable biological functions as well as the network of signaling pathways that may be important in prostate cancer. Novel phosphorylation sites of β -catenin, HDAC2, and USP10 which are known to interact with AR and other cancer-related molecules could be further characterized to elucidate molecular mechanism involved in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	Androgen Receptor
CK	Casein Kinase
GO	Gene Ontology
GSK-3	Glycogen Synthase Kinase 3

HDAC	Histone Deacetylase
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
MudPIT	Multidimensional Protein Identification Technology
MW	Molecular Weight
NaDOC	Sodium Deoxycholate
pS	Phosphoserine
PSA	Prostate-Specific Antigen
pT	Phosphothreonine
PTM	Post-translational Modification
pY	Phosphotyrosine
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture

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Fig. 1. Outline of the experimental strategy for identification of phosphoproteome of LNCaP cells

Flowchart of the experimental scheme of phosphoproteome analysis. NaDOC, sodium deoxycholate; TiO₂, titanium dioxide; HPLC, high performance liquid chromatography.

Fig. 2. Classification of identified phosphoproteins according: (A) to biological process; (B) molecular function; (C) cellular component in NaDOC; and (D) cellular component in urea All identified phosphoproteins expressed in LNCaP cells were analyzed and classified in different categories using Gene Ontology (GO). Pie charts represent the most frequent biological functions and processes (A) and molecular function (B) associated with phosphoproteins. Major cellular component of identified phosphoproteins are represented with a slightly different portion of membrane compartments (plasma membrane, ER membrane, and membrane fraction) between the different conditions of digestion, NaDOC (C) and Urea (D).

Fig. 3. Signaling pathways involving identified phosphoproteins

Different signaling pathways hits are represented by PANTHER analysis from all identified phosphoproteins in LNCaP cells. The major signaling pathway was Wnt, followed by cadherin, Rho GTPase, heterotrimeric G-protein and Gi alpha and Gs alpha mediated signaling pathways.

Fig. 4. MS/MS spectrum of phosphopeptides of AR-interacting proteins

Representative MS/MS spectrum of phosphopeptides identified for HDAC2, USP10, and β -catenin and the protein kinase motif of each peptide predicted using PHOSIDA. MS/MS of the precursor ion of m/z 773.2621(2+) of HDAC2, confirming the indicated peptide sequence, IACDEEFpSDpSEDEGEGGR with predicted CK2 motif (A), m/z 1036.4176 (2+) of USP10, confirming the indicated peptide sequence, NHSVNEEEQEEQGEGpSEDEWEQVGPR with predicted CK2 motif (B) and m/z 620.9515 (2+) of β -catenin, confirming the indicated peptide sequence, RTpSMGGRTQQQFVEGV with predicted PKA motif (C).

Fig. 5. Ingenuity pathway analysis of cancer related phosphoproteins

Sixty-one genes reported in the relationship with cancer are represented from all identified phosphoproteins in LNCaP cells. Interactions, localization and prostate cancer function are shown. Direct and indirect involvement in cancer represented in blue and gray lines, respectively.

Table 1

Comparison of statistical parameters of phosphoproteins identified using sodium deoxycholate (NaDOC) or urea in trypsin digestion

Parameter	Method	
	NaDOC ^c	Urea
Total identified unique peptides	125	127
Total identified unique proteins	90	89
% of phosphoproteins with positive GRAVY ^a values	6.7	5.6
% of phosphopeptides with 0 (1) missing cleavage sites	58.4 (41.6)	55.9 (44.1)
Average pI	6.75	6.70
Molecular weight ^b < 20 kDa	5.60%	8.66%
20 – 40 kDa	25.60%	28.35%
40 – 60 kDa	12.80%	14.17%
60 – 80 kDa	12.80%	10.24%
80–100 kDa	9.60%	7.87%
100–120 kDa	11.20%	15.75%
120–140 kDa	5.60%	4.72%
140–200 kDa	4.00%	4.72%
> 200 kDa	12.80%	5.51%

^a GRAVY, Grand Average of Hydropathy value;

^b Theoretical molecular weight of unique phosphoproteins calculated using ProtParam (<http://www.expasy.org/tools/protparam.html>) was distributed and compared between two different surfactants containing enzyme digestion;

^c NaDOC, sodium deoxycholate

Table 2

Total number of proteins identified per number of unique peptides

No. of peptides identified ^a	NaDOC ^b	Urea
1	67	66
2	16	13
3	3	7
4	2	2
5	2	1
Total	90	89

^aTotal number of unique phosphoproteins were counted and compared between two different surfactants containing enzyme digestion;

^bNaDOC, sodium deoxycholate

Table 3

Distribution of predicted kinase motifs of identified phosphopeptides

Kinase	%	Kinase	%
CK1	28.14%	Aurora	1.71%
CK2	22.60%	EGFR	1.71%
GSK3	9.59%	SRC	1.28%
CAM2K	8.32%	AKT	0.64%
NEK6	7.68%	ALK	0.64%
PKA	5.12%	ABL	0.21%
ERK	3.20%	Aurora-A	0.21%
CDK1	2.99%	CHK1	0.21%
PLK1	2.99%	PKD	0.21%
CDK2	2.35%	PLK	0.21%

Table 4

Identified proteins known to interact with AR as phosphorylated forms

Protein Acc. No. ^a	Protein Name	Gene Name	Functions ^b	coA/ coR ^c	Ref ^d
IP100289601	Histone deacetylase 2	HDAC2	Deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Transcriptional regulation, cell cycle progression and developmental events. Forms transcriptional repressor complexes by associating with MAD, SIN3, YY1 and N-COR. Interacts in the late S-phase of DNA-replication with DNMT1 in the other transcriptional repressor complex composed of DNMT1, DMAP1, PCNA, CAF1.	coR	54, 76
IP100291946	Ubiquitin carboxyl-terminal hydrolase 10	USP10	Ubiquitin specific protease are required to remove ubiquitin from specific proteins or peptides to which ubiquitin is attached.	coA	57
IP100017292	Isoform 1 of Catenin beta-1	CTNNB1	Regulation of cell adhesion and signal transduction through the Wnt pathway.	coA	56, 77-79

^aProtein Acc. No., Protein accession number searched in IPI_human database;

^bFunctions, molecular functions of proteins referred in SwissProt;

^ccoA/ coR, Co-activator and Co-repressor in androgen receptor (AR) signaling;

^dRef, References described about proteins

Table 5

Data from mass spectrometry analysis including phosphorylation sites and physical parameters of identified phosphorylated AR interacting proteins

Acc. No. ^a	Protein Name	AA ^b	Mw ^c (kDa)	pI ^d	GRAVY ^e	Surfactants	Pep_score ^f	miss	Peptide sequences ^g	P-sites ^h
IP100289601	Histone deacetylase 2	488	55.36	5.59	-0.718	Urea	52.76	1	R.IACDEEFSDEGE GGRR .N	S422, S424
IP100291946	Ubiquitin carboxyl-terminal hydrolase 10	798	87.13	5.19	-0.449	Urea NaDOC	111.35 80.02	0 0	K.NHSVN EEEEQEEQEGSEDEWEQV GPR.N K.NHSVN EEEEQEEQEGSEDEWEQV GPR.N	S576 S576
IP100017292	Isoform 1 of Catenin beta-1	781	85.49	5.53	-0.175	Urea NaDOC NaDOC	66.32 80.26 129.00	1 0 0	R.RTSMGGTQQQFVEGV R .M R.TSMGGTQQQFVEGV R .M R.TSMGGTQQQFVEGV R .M	S552 T551 S552

^a Acc. No., Protein accession number searched in IPI_human database;

^b AA, The number of amino acid of protein;

^c Mw, Theoretical molecular weight;

^d pI, Theoretical isoelectric point;

^e GRAVY, Grand Average of Hydropathy value;

^f pep_score, Peptide score acquired from Mascot search;

^g Peptide sequence, Identified phosphopeptide sequences (the modification of phosphorylation on serine and threonine residue as well as oxidation on methionine residue were underlined);

^h P-sites, The position of phosphorylation sites in protein sequence