Proteomic Analysis of Microvesicles Released by the Human Prostate Cancer Cell Line PC-3

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Cancer biomarkers are invaluable tools for cancer detection, prognosis, and treatment. Recently, microvesicles have appeared as a novel source for cancer biomarkers. We present here the results from a proteomic analysis of microvesicles released to the extracellular environment by the metastatic prostate cancer cell line PC-3. Using nanocapillary liquid chromatography-tandem mass spectrometry 266 proteins were identified with two or more peptide sequences. Further analysis showed that 16% of the proteins were classified as extracellular and that intracellular proteins were annotated in a variety of locations. Concerning biological processes, the proteins found in PC-3 cell-released microvesicles are mainly involved in transport, cell organization and biogenesis, metabolic process, response to stimulus, and regulation of biological processes. Several of the proteins identified (tetraspanins, annexins, Rab proteins, integrins, heat shock proteins, cytoskeletal proteins, 14–3-3 proteins) have previously been found in microvesicles isolated from other sources. However, some of the proteins seem to be more specific to the vesicular population released by the metastatic prostate cancer PC-3 cell line. Among these proteins are the tetraspanin protein CD151 and the glycoprotein CUB domain-containing protein 1. Interestingly, our results show these proteins are promising biomarkers for prostate cancer and therefore candidates for clinical validation studies in biological fluids. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.012914, 1–11, 2012.

Prostate cancer is one of the most frequent cancer types in men with 679,000 diagnoses and 220,000 deaths worldwide each year (1). Prostate tumorigenesis is still poorly understood, although several important molecular mechanisms for prostate cancer development such as androgen receptor signaling have been discovered (2). Prostate-specific antigen has been extensively used as a prostate cancer marker. However, prostate-specific antigen has serious limitations as a tumor marker since its use has lead to overdiagnosis and overtreatment of the disease (3). Furthermore, since prostate cancer often grows slowly, markers that can provide information about cancer aggressiveness are required. Several promising markers such as prostate stem cell antigen, α-methylacyl coenzyme A racemase, early prostate cancer antigen, human kallikrein 2, hespin and glutathione S-transferase are currently undergoing validation (4).

It has been shown that a number of different cell types release microvesicles to the extracellular environment (5–9). Interestingly, cancer cells seem to release more microvesicles that control cells. It has for example been shown that plasma samples of patients with advanced lung cancer, ovarian cancer, and prostate cancer had higher levels of microvesicles compared with control patients (10–12). Microvesicles are able to affect neighboring cells in various ways, for example by inducing intracellular signaling or by transferring different molecules such as proteins, mRNAs, or microRNAs to cells (13, 14). Concerning cancer, microvesicles have been suggested to contribute to cancer cell survival, invasiveness, and metastases (15, 16). Furthermore, microvesicles are a source of cancer biomarkers because they carry tumor-related molecules (16–18). Promising results show that plasma samples from ovarian cancer patients contain claudin-4-containing exosomes (19) and that plasma from melanoma patients contain high levels of microvesicles expressing CD63 and caveolin-1 (20).

In addition to blood plasma, microvesicles have been found in many biological fluids such as urine, seminal fluid, saliva, tear fluid, breast milk, and amniotic fluid. However, body fluids contain a mixture of microvesicles originating from different cells types that makes difficult the analysis and interpretation of proteomic studies. Therefore, several groups have performed proteomic studies on cell lines originating from specific cancer diseases (9, 21–23).

There has recently been a considerable increase in the number of articles published on microvesicles (for reviews see (16, 18, 24–28). Different names (microvesicles, exosomes, prostasomes, ectosomes, exosome-like vesicles, membrane particles), isolation protocols, and vesicle sizes can be found in the literature based on the source of microvesicles and/or release mechanism. Scientists working in this field are trying to get consensus on these issues. Even though there are still many unanswered questions, microvesicles seem to be released by two main mechanisms: direct budding from the
plasma membrane (24) and fusion of multivesicular bodies (MVB) with the plasma membrane, a process that leads to the release of the internal vesicles contained in the MVB (27). Microvesicles that originate from the plasma membrane are often referred to as shedding vesicles. These vesicles are 100–1000 nm in diameter, sediment at 10,000 × g (25) and are often secreted when cells are submitted to stress conditions. However, microvesicles that originate from MVB, exosomes, typically have a size diameter of 50–100 nm and sediment at 100,000 × g (25). It should be mentioned that cells may contain different types of MVBs, and that there may be a specific MVB population given rise to exosomes (27).

In this study the metastatic prostate cancer cell line PC-3 was used. The microvesicles pelletted at 100,000 × g from the culture medium of these cells have previously been referred as prostasomes (8, 29), a term used to name vesicles released by prostate cells (30, 31). There is strong evidence that most microvesicles released from PC-3 cells are secreted in a similar way as exosomes (8, 29). However, because, at the moment, we can not be sure whether there is a fraction of vesicles released by another mechanism, we will here be referring to these vesicles with the more general term of microvesicles.

The main goal of this study is to identify proteins in microvesicles released from PC-3 cells that can potentially be used as prostate cancer biomarkers. To obtain the detailed protein composition of these microvesicles, a nanocapillary liquid chromatography–tandem mass spectrometry (nano LC-MS/MS) proteomic analysis was performed. This analysis may also provide us with information about the mechanism of release of these vesicles.

EXPERIMENTAL PROCEDURES

**Materials**—Dithiothreitol (GE Healthcare, Oslo, Norway), iodoacetamide (Sigma-Aldrich Norway), trypsin porcine from (Promega, Madison, WI), nC18 Empore 3 m Extraction Disks (Agilent Technologies, Palo Alto, CA), antibody to caveolin-1 (BD Biosciences, San Diego, CA), antibody to CUB domain-containing protein 1 (CDCP1) (R&D Systems, Abingdon, UK), antibodies to CD147 and CD151 (Abcam, Cambridge, UK), antibody to calreticulin (Stressgen, Enzo Life Sciences), antibody to MOC31 (anti-EpCAM) (IQ Products, Groningen, The Netherlands), DMEM/F-12 (1:1 Mix of DMEM and Ham’s F-12) medium, RPMI 1640 medium and keratinocyte–serum free medium kit with l-glutamine, epithelial growth factor and bovine pituitary extract were from Invitrogen, (Invitrogen Dynal, Norway). The immunomagnetic M450 Dynabeads (diameter 4.5 μm) were from Invitrogen (Oslo, Norway). Bicinchoninic acid protein assay kit and immunomagnetic M450 Dynabeads (diameter 4.5 μm) were from Pierce (Rockford, IL).

**Cell Culture**—The epithelial human prostate cancer cell line PC-3 (32) obtained from the American Type Culture Collection was maintained in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s modified Eagle’s medium supplemented with 7% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The epithelial human prostate cell line RWPE-1 was obtained from the American Type Culture Collection and grown in keratinocyte serum-free medium supplemented with bovine pituitary extract (0.05 mg/ml) and EGF (5 ng/ml), 100 units/ml penicillin, and 100 μg/ml streptomycin. The nonmetastatic prostate cancer cell line LNCaP was grown in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in an atmosphere of 5% CO2/95% air.

**Microvesicle Isolation**—PC-3 cells were grown in serum-free cell culture medium for 3 days and microvesicles were isolated from the culture medium as previously described (8). Serum-free medium was used to avoid contamination with vesicles contained in serum. The culture medium was centrifuged to remove cell debris first at 400 × g for 10 min and then at 10,000 × g for 30 min. Vesicles were then collected by ultracentrifugation at 100,000 × g for 2 h in a SW40 or SW28 rotor, washed with a large volume of phosphate-buffered saline, and then concentrated by ultracentrifugation at 100,000 × g for 2 h in a SW40 rotor first, and then in a TLA 120.1 rotor. For mass spectrometry analysis microvesicles were isolated from 5–7 × 107 cells.

**Protein Determination**—PC-3 cells and microvesicles were lysed in lysis buffer (25 mm Tris-HCl, 125 mm NaCl, 5 mm EDTA, 1% Triton X-100, SDS 0.1%, deoxycholate 2 g/l, pH 7.4) in the presence of a protease inhibitor mixture. Then, the protein content was determined using a bicinchoninic acid protein assay kit according to the manufacturer’s instructions.

**SDS-PAGE**—Pelleted microvesicles were directly solubilized in loading buffer. Whole-cell lysates were solubilized in lysis buffer (25 mm Tris-HCl, 125 mm NaCl, 5 mm EDTA, 1% Triton X-100, SDS 0.1%, deoxycholate 2 g/l, pH 7.4) in the presence of a protease inhibitor mixture. Sample buffer was added to the cell lysates after removal of insoluble material. Microvesicles and lysate samples were then subjected to SDS-PAGE. Microvesicle samples destined for LC-MSMS were run in 4–20% gradient gels, stained with Coomassie Blue and cut in pieces.

**Peptide Generation**—For generation of peptides, microvesicle proteins contained in gel pieces were treated as followed. First, possible disulfide bridges between cysteines were broken by reduction with dithiothreitol and alkylated with iodoacetamide to prevent oxidation and formation of new disulfide bridges. Proteins were then digested in-gel by trypsin for 16 h at 37 °C. The protease activity was stopped by acidification using 2% formic acid. Samples were then filtered in a homemade nC8 StageTip (Stop and go extraction Tip, using nC8 Empore 3 M Extraction Disks column. The sample was eluted through reduced to about 10 μl with 0.1% TFA and nanocapillary liquid chromatography–tandem mass spectrometry (nano LC-MS/MS) proteomic analysis was performed. This analysis may also provide us with information about the mechanism of release of these vesicles.
using [Glu]-fibrinopeptide B (monoisotopic mass: 1569.67; amino acid sequence: EGVNDNEEGFSSAR. The calibration utilized fragment masses of this peptide ([M + 2H]2+, m/z 785.8426, z = 2). The MS survey scan was acquired on the range m/z 300–1500 Da with a scan time of 0.9 s and an interscan delay of 0.1 s. The maximum number of ions selected for MSMS was 3 and m/z 50 to 2000 with a scan time of 0.9 s and an interscan delay of 0.1 s was used for MS/MS acquisition. Bovine serum albumin was digested together with samples as a control of method and instrumentation. A specific peak (777.8 Da) was closely monitored and no mass errors and chromatographic malfunctioning were discovered. The mass spectra of fragmented peptides were smoothed (Savitzky Golay), centered, and combined in a merge file. All the MSMS spectra were used to search the UniProt database (www.uniprot.org, 16-Nov-2011, 23224 reviewed sequences/Homo sapiens, reversed decoy sequences were added) with in-house Mascot 2.3.0 (www.matrixscience.com). Searches were performed with a tolerance on mass measurement of 0.3 Da. The Mascot results were analyzed with PeptideShaker ver. 0.12.2 (http://peptide-shaker.googlecode.com). Proteins and peptides were identified with 1% FDR. The false discovery rate (FDR), was calculated as the percentage of positive hits in the decoy database versus the target database both for proteins and peptides. This resulted in 1836 peptides and 416 proteins. Proteins of interest identified with one peptide only were manually verified. LC-MSMS analysis was done at PROBE Laboratory, Proteomic Unit at the University of Bergen, Norway. The bioinformatics tool ProteinCenter 3.8 (Thermo Fisher Scientific, Odense, Denmark) was used to analyze the results of this proteomics study.

**Microvesicle Immunoisolation with EpCAM-Beads**—M450 Dynabeads coated with sheep anti-mouse antibodies were coated with the anti-EpCAM antibodies as previously described (33). Four micrograms of microvesicles were incubated with 10 million EpCAM-dynabeads overnight at 4 °C with rotation. Bead-bound microvesicles were isolated on a magnet and EpCAM-positive microvesicles bound to the beads were eluted by boiling in SDS-sample buffer.

**Western Blot**—Normally, 1–3 µg of vesicles were loaded on SDS-PAGE gels and compared with equal amounts of lysates. In some occasions higher amounts of lysates were required to detect specific proteins by Western blot. Pelleted microvesicles were directly solubilized in loading buffer. SDS-PAGE gels were transferred to Immobilon-P membranes and then the membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 in PBS and incubated with the indicated primary antibody. The membranes were then washed three times for at least 5 min with 0.1% Tween 20 in phosphate-buffered saline, and then incubated with secondary antibodies coupled to horseradish peroxidase. Finally, the membranes were washed three times for at least 5 min and developed using an enhanced chemiluminescence detection kit.

**RESULTS AND DISCUSSION**

**Microvesicles Released by PC-3 Cells**—PC-3 cells release a vesicle population that is pelleted from conditioned medium at 100,000 × g. These vesicles have previously been collected from serum-free culture medium after 16–24 h (8, 29). Because high amounts of microvesicle proteins are required for proteomic analysis (25–50 µg), an experiment was performed to investigate whether longer collection times would result in higher amounts of microvesicle released proteins. Collection of vesicles was started 1, 2, or 3 days after plating and continued for 3, 2, and 1 day respectively. Similar number of cells were collected at the end of the experiment and a trypan blue exclusion test showed that the cells were viable (data not shown). The release of microvesicles was measured by quantifying the amount of caveolin-1, a protein that is known to be present in these vesicles (8) and by measuring the total amount of protein in the vesicles. Both the amount of caveolin-1 (Fig. 1A) and the total amount of protein (data not shown) in the vesicles increased when the collection time was increased. Control experiments showed that the amount of caveolin-1 in cell lysates was similar in the three conditions (Fig. 1A).

**Nano LC-MS/MS Proteomic Analysis of PC-3 Cell-released Microvesicles**—In order to determine the protein composition of vesicles released to the extracellular environment by PC-3 cells, a proteomic analysis using nanocapillary liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) was performed. Proteins present in the microvesicles were separated by SDS-PAGE using 4–20% gels (Fig. 1B) that were then sliced. Proteins were digested in-gel with porcine trypsin to obtain peptides that were fragmented using nano-LC-MS/MS. The obtained MSMS spectra were used to search the UniProt database (16-Nov-2011, 23224 reviewed sequences/Homo sapiens) with Mascot 2.3.0. The Mascot results were analyzed with PeptideShaker ver. 0.12.2. The database search resulted in the identification of 1836 peptides (supplemental Fig. S1) and 416 proteins with 1% FDR (see Experimental Procedures). Of those proteins, 266 proteins were identified with two or more unique peptide sequences (supplemental Figs. S2 and S3). The annotated spectra of some specific proteins identified with only one peptide that are later mentioned in the manuscript are shown in the supplementary information (supplemental Fig. S4).
To determine the reproducibility of the method an independent experiment was performed. Database search resulted in this case in 891 unique peptide sequences and 255 proteins. As shown in Fig. 1C, 214 proteins were found in both experiments thus showing the reproducibility of the method. The fact that a lower number of proteins was found in the second data set may be because of the lower amount of total protein that was analyzed.

**Analysis of Proteins Contained in PC-3 Cell-released Microvesicles**—We further analyzed the 266 proteins identified with two or more peptide sequences (supplemental Figs. S2 and S3) with ProteinCenter 3.8 (Thermo Fisher Scientific, Odense, Denmark), a web-based data interpretation tool that facilitates the comparison and interpretation of data sets. Proteins were classified based on Gene Ontology slim (GOslim) annotations for cellular localization and biological process specifically defined for ProteinCenter. It should be kept in mind that GO annotations often provide several locations and functions for a single protein. From the 266 proteins analyzed, 977 annotations for GOslim cellular components and 1398 annotations for GOslim biological processes were obtained. Fig. 2A shows GOslim annotations for cellular localization. 42 proteins (16%) were annotated as extracellular. Furthermore, only 49 proteins (18%) were predicted to contain signal peptides (PrediSi, PREDIction of SIgnal peptides software) in agreement with the idea that microvesicle proteins are not released by the classical secretory pathway. Furthermore, PC-3 cell-derived microvesicles were not enriched in plasma membrane proteins in agreement with the idea that these vesicles do not originate to a large extent from the plasma membrane. Intracellularly, the proteins were annotated to the cytosol or to different organelles such as nucleus, endosomes, or endoplasmic reticulum. Interestingly, a high percentage of proteins was annotated to the category cytoskeleton (Fig. 2A and Table I). Thus, similarly to other extracellular vesicular populations, proteins found in PC-3 cell
Microvesicles are intracellularly associated to a variety of cellular organelles (9).

In Fig. 2B the GOslim annotations for biological processes of the proteins present in PC-3 cell microvesicles are shown. The 5 top annotations for proteins were regulation of biological process, transport (Table II), metabolic process, response to stimulus and cell organization and biogenesis. Furthermore, pathways annotations were found using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, an information resource that contain wiring diagrams of molecular interactions and reaction networks (34). Table III shows the KEGG pathways to which 13 or more proteins in PC-3 cell microvesicles were annotated. As it could be expected from the high number of cytoskeleton and cytoskeletal associated proteins, many proteins in PC-3 cell microvesicles were annotated to the “regulation of actin cytoskeleton” pathway. Furthermore, several proteins were annotated to the “focal adhesion” and the “tight junction” pathways. Interestingly, the annotation “pathways in cancer” and “MAPK signaling pathway,” a pathway that plays a critical role in cancer are also found in Table III. Finally, 17 proteins were annotated to the KEGG pathway “endocytosis.”

The MS approach used in this proteomic study is not quantitative, but can give us a rough idea of protein abundance based on the number of peptides identifying each protein. In Table IV, the 43 proteins that were identified with highest number of unique peptides (12 or more) are listed. It is remarkable that there are several integrins (integrin beta-4, integrin alpha-2, integrin beta-1, integrin alpha-3, integrin alpha-6) and several cytoskeletal associated proteins that

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### Table I

| Protein name                      | Acc. no |
|----------------------------------|---------|
| Actin, aortic smooth muscle      | P62736  |
| Actin, cytoplasmic 1             | P60709  |
| Actin-related protein 2          | P61160  |
| Actin-related protein 2/3 complex subunit 2 | O15144  |
| Actin-related protein 2/3 complex subunit 3 | O15145  |
| Actin-related protein 2/3 complex subunit 4 | P59998  |
| Actin-related protein 2/3 complex subunit 5-like protein | Q9BPX5  |
| Alpha-actinin-1                  | P12814  |
| Alpha-actinin-4                  | O43707  |
| Cathein beta-1                   | P35222  |
| Cathein delta-1                  | O60716  |
| Coactosin-like protein           | Q14019  |
| Cofilin-1                        | P23528  |
| Destrin                          | P60981  |
| Dynein heavy chain               | Q14204  |
| Ezrin                            | P15311  |
| F-actin-capping protein subunit alpha-1 | P52907  |
| Fascin                           | Q16658  |
| Filamin-A                        | P21333  |
| Filamin-B                        | O75369  |
| Filamin-C                        | Q14315  |
| Junction plakoglobin             | P14923  |
| Moesin                           | P26038  |
| Myosin Ib                        | O43795  |
| Myosin Ic                        | O00159  |
| Myosin Id                        | O94832  |
| Myosin-9                         | P33579  |
| Myristoylated alanine-rich C-kinase substrate | P29966  |
| Nck-associated protein 1          | Q9Y2A7  |
| Plastin-2                        | P13796  |
| Plastin-3                        | P13797  |
| Plectin-1                        | Q15149  |
| Plexin-B2                        | O15031  |
| Profilin-1                       | P07737  |
| Radixin                          | P35241  |
| Septin-2                         | Q15019  |
| Talin-1                          | Q9Y490  |
| Tropomyosin alpha-3 chain         | P06753  |
| Tubulin alpha-1B chain           | P68363  |
| Tubulin beta chain               | P07437  |
| Tubulin beta-4 chain             | P04350  |
| Vinculin                         | P18206  |

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### Table II

| Protein name                      | Acc. no |
|----------------------------------|---------|
| ADP-ribosylation factor 3        | P61204  |
| ADP-ribosylation factor 4        | P18085  |
| ADP-ribosylation factor 5        | P84085  |
| ADP-ribosylation factor 6        | P62330  |
| Alpha-soluble NSF attachment protein | P54920  |
| Annexin A1                      | P04083  |
| Annexin A2                      | P07355  |
| Annexin A3                      | P12429  |
| Annexin A5                      | P08758  |
| Annexin A6                      | P08133  |
| Annexin A7                      | P20073  |
| Annexin A11                     | P50995  |
| Clathrin heavy chain 1           | Q00610  |
| EH-domain-containing protein 1   | Q9HM49  |
| EH-domain-containing protein 4   | Q9H223  |
| Heat shock 70 kDa protein 1A/1B  | P08107  |
| Myoferlin                       | Q9NZM1  |
| Programmed cell death 6-interacting protein | Q8WUM4  |
| Rab-1A                          | P62820  |
| Rab-1B                          | Q9H0U4  |
| Rab-5A                          | P20339  |
| Rab-5B                          | P61020  |
| Rab-7a                          | P51149  |
| Rab-8A                          | P61006  |
| Rab-8B                          | Q92930  |
| Rab-10                          | P61026  |
| Rab-11A                         | P62491  |
| Rab-12                          | Q61222  |
| Rab-13                          | P51153  |
| Rab-14                          | P61106  |
| Rab-22A                         | Q9UL26  |
| Rab-27B                         | O00194  |
| Rab-35                          | Q15286  |
| Syntaxin-4                      | Q12846  |
| Syntaxin-binding protein 3      | Q00186  |
| Vesicle-associated membrane protein 8 | Q9BV40  |

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**Proteomics of PC-3 Cell Microvesicles**

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facilitate binding of integrins to actin (talin, vinculin, alpha-actinin, and filamin) in the list. Integrins are heterodimeric cell surface receptors for extracellular matrix proteins (35). Ligand binding causes integrin clustering and recruitment of actin filaments to the integrin cytoplasmic domains. The importance of integrins in cancer is well-known because of their function in cell adhesion, migration, proliferation and cell survival (36), and prostate cancer is not an exception (37). Integrins are commonly found in exosomes where they seem to be involved in anchoring the vesicles to the extracellular matrix (38). Table IV also contains several transport proteins (i.e., annexin A2, clathrin heavy chain, EH-domain-containing protein 1 and 4), enzymes (i.e., aminopeptidase, pyruvate kinase), heat shock proteins and proteins involved in signal transduction (i.e., 14–3-3 proteins, guanine nucleotide-binding proteins, ephrin type-A receptor 2).

**PC-3 Cell Microvesicles and Exosomes**—There are several studies about the protein composition of exosomes, vesicles released after fusion of MVB with the plasma membrane (27). Therefore, we decided to compare the protein composition of PC-3 cells-released microvesicles and exosomes. Exosomes originating from different cell types have a common set and a specific set of proteins. Common proteins found in exosomes are integrins, heat shock proteins, tetraspanins, proteins involved in vesicular transport and cellular signaling, and cytoskeletal proteins. Integrins, heat shock proteins, and signaling proteins are widely represented in PC-3 cell-released microvesicles (supplemental Figs. S2 and S3). Also several tetraspanin proteins, membrane proteins characterized by the presence of four hydrophobic domains, were found in these vesicles: CD9, CD63, CD81, and CD151. In addition, tetraspanin9 and tetraspanin15 were identified with only one peptide (supplemental Fig. S4). Typical cytoskeletal and vesicular transport proteins in PC-3 microvesicles have been listed in Table I and Table II respectively. Table II includes for example clathrin and several members of the Rab, annexin and ARF family of proteins. These proteins are involved in one or several transport mechanism along the endocytic and/or exocytic pathway and are not specific for microvesicle release. However, there are probably important regulators of exosome

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**TABLE III**

KEGG pathways to which 13 or more proteins in PC-3 cells microvesicles were annotated

| KEGG ID   | KEGG Pathway                        | No. proteins |
|-----------|-------------------------------------|--------------|
| hsa04810  | Regulation of actin cytoskeleton     | 30           |
| hsa04510  | Focal adhesion                       | 22           |
| hsa05870  | Leukocyte transendothelial migration | 18           |
| hsa04144  | Endocytosis                          | 17           |
| hsa04980  | Pathways in cancer                   | 16           |
| hsa04722  | MAPK signaling pathway               | 15           |
| hsa04662  | Chemokine signaling pathway          | 14           |
| hsa05130  | Pathogenic Escherichia coli infection| 14           |
| hsa04145  | Phagosome                            | 13           |
| hsa04360  | Axon guidance                        | 13           |
| hsa05100  | Bacterial invasion of epithelial cells| 13           |

* KEVG: Kyoto Encyclopedia of Genes and Genomes.

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**TABLE IV**

Proteins identified with 12 or more unique peptides in PC-3 cells microvesicles

| Unique peptides | Accession number | Protein name                                      |
|-----------------|------------------|--------------------------------------------------|
| 39              | P16144           | Integrin beta-4 (CD104 antigen)                  |
| 33              | P18206           | Vinculin                                         |
| 31              | P15144           | Aminopeptidase N (CD13)                          |
| 30              | P12814           | Alpha-actinin-1                                  |
| 28              | P17301           | Integrin alpha-2 (CD49b antigen)                 |
| 27              | P05556           | Integrin beta-1 (CD29 antigen)                   |
| 25              | P26006           | Integrin alpha-3 (CD49c antigen)                 |
| 24              | P21333           | Filamin-A                                        |
| 24              | P26038           | Moesin                                           |
| 23              | O75369           | Filamin-B                                        |
| 23              | P23229           | Integrin alpha-6 (CD49f antigen)                 |
| 22              | O60716           | Catenin delta-1                                  |
| 22              | P05023           | Sodium/potassium-transporting ATPase subunit alpha-1 |
| 22              | P07355           | Annexin A2                                       |
| 22              | Q15149           | Plectin                                          |
| 19              | Q9H4M9           | EH domain-containing protein 1                   |
| 18              | P60709           | Actin, cytoplasmic 1                             |
| 18              | O00159           | Myosin-ic                                        |
| 18              | O43854           | EGF-like repeat and discoidin I-like domain-containing protein 3 |
| 18              | P11142           | Heat shock cognate 71 kDa protein                |
| 18              | P29317           | Ephrin type-A receptor 2                         |
| 16              | P62736           | Actin, aortic smooth muscle                       |
| 16              | O43707           | Alpha-actinin-4                                  |
| 16              | P14923           | Junction plakoglobin                             |
| 16              | P63104           | 14-3-3 protein zeta/delta                        |
| 15              | P27348           | 14-3-3 protein theta                             |
| 15              | P35222           | Catenin beta-1                                   |
| 15              | Q09666           | Neuroblast differentiation-associated protein AHNAK |
| 14              | O43795           | Myosin-ib                                        |
| 14              | P07900           | Heat shock protein HSP 90-alpha                  |
| 14              | P14618           | Pyruvate kinase isozymes M1/M2                   |
| 14              | P31946           | 14-3-3 protein beta/alpa                         |
| 13              | P04899           | Guanine nucleotide-binding protein G(i) subunit alpha-2 |
| 13              | P08238           | Heat shock protein HSP 90-beta                   |
| 13              | P15311           | Ezrin                                            |
| 13              | P62937           | Peptidyl-prolyl cis-trans isomerase A            |
| 13              | Q7L576           | Cytoplasmic FMR1-interacting protein 1           |
| 13              | Q9H223           | EH domain-containing protein 4                   |
| 12              | P35241           | Radixin                                          |
| 12              | P62258           | 14-3-3 protein epsilon                           |
| 12              | Q00610           | Catenin heavy chain 1                            |
| 12              | Q9H5V8           | CUB domain-containing protein 1                  |
| 12              | Q9P265           | Disco-interacting protein 2 homolog B           |
release among these proteins. In fact, several Rab proteins, Rab11 (39), Rab27 (40), and Rab35 (41), have already been shown to regulate exosome release. Interestingly, EH-domain-containing proteins 1 and 4 were identified with a high number of peptides (Table IV). These proteins belong to the EPS15 homology (EH) domain-containing proteins family and have previously been involved in early endosomal transport and recycling (42, 43). Moreover, myoferin, a protein recently implicated in the endocytic pathway (44) that binds to EH domain-containing family members (45) is also present in PC-3 microvesicles. Further investigations are required to determine whether these proteins regulate the release of microvesicles in PC-3 cells.

Because the vesicles released by PC-3 cells may proceed from MVB, the presence in PC-3 cell-derived microvesicles of proteins normally located in MVB/lysosomes was then investigated. In fact two MVB associated proteins, CD63 and the endosomal sorting complex required for transport (ESCRT) protein Alix (programmed cell death 6-interacting protein), are commonly found in exosomes and have been considered as exosome markers (27). Both CD63 and Alix are found in PC-3 microvesicles. Furthermore, several ESCRT (vps28, CHMP1B, CHMP4B) were identified with only one peptide (supplemental Fig. S4).

The proteins identified in PC-3 cell microvesicles proteins where then compared with the proteins found in Exocarta 3.2 (http://exocarta.ludwig.edu.au), a compendium for proteins identified in exosomes (46). First, the presence in PC-3 cell microvesicles of the top 25 proteins often identified in exosomes was investigated. Only two proteins (major histocompatibility complex class II and syntenin) were not found in PC-3 cell-derived microvesicles. Moreover, only 6% of the proteins in PC-3 cell microvesicles were not found in Exocarta. However, this number may be smaller since these proteins may have been identified in studies not included in Exocarta. It should also be mentioned that several of these proteins belong to families of proteins that are described in Exocarta. In conclusion, the fact that most of the proteins in PC-3 cell microvesicles, including several MVB proteins considered as exosomal markers, have previously been identified in exosomes is in agreement with the idea that microvesicles released from PC-3 cells are exosomes released by prostate cells.

Identification of Cancer Relevant Proteins in PC-3 Cell Microvesicles—We decided next to investigate whether there were proteins in PC-3 microvesicles that could be potentially useful as biomarkers for prostate cancer. In particular, three proteins were considered especially interesting because of their described involvement in prostate cancer: the membrane glycoprotein CUB domain-containing protein 1 protein (CDCP1), the tetraspanin CD151, and CD147 (also named basigin).

CDCP1 was investigated first since this protein was not found in Exocarta, and it could then be more specific of prostate cancer released microvesicles. CDCP1 has been described as a tumor marker (47), and it has been proposed to function as an antiapoptotic molecule that facilitates tumor cell survival during metastasis (48). Furthermore, it has been shown that a monoclonal antibody against CDCP1 inhibits metastasis in a prostate cancer model (49). As shown in Fig. 3A, the presence of this protein in microvesicles was confirmed by Western blot. The CDCP1 band appears at a molecular weight (between the molecular markers 100 and 150 kDa) that corresponds to the glycosylated form of the protein. Furthermore, when equal amounts (2 μg) of protein from microvesicles and cell lysates were analyzed by SDS-PAGE and Western blot, CDCP1 was only weakly detected in the lysates (Fig. 3A), thus showing that CDCP1 is enriched in microvesicles. Higher amounts of lysates (10 μg) were required to better detect CDCP1 in PC-3 lysates (Fig. 3A). As a control, the presence of the endoplasmic reticulum protein calreticulin was investigated. As expected, this protein was mainly detected in the lysates of both PC-3 and RWPE-1 cells and not significantly in the released microvesicles (Fig. 3B).

**Fig. 3. Analysis of CDCP1 as a potential prostate cancer biomarker.** Microvesicles were isolated from PC-3 and RWPE-1 cells as indicated under “Experimental Procedures.” The amounts of protein loaded per lane are indicated in the figure. CDCP1 (A) and calreticulin (B) were then detected in lysates (Lys) and microvesicles (Ves) by Western blot. Bands at the predicted molecular weight for CDCP1 (140 kDa) and calreticulin (63 kDa) were observed. C, Microvesicles were isolated from conditioned media of PC-3 cells and LNCaP as indicated under “Experimental Procedures.” Sample buffer was added and samples were loaded on SDS-PAGE gels. The presence of CDCP1 in the samples was analyzed by Western blot. D, Microvesicles were isolated from the conditioned media of PC-3 cells and immunosolated by EpCAM-dynabeads. Bound microvesicles were lysed in sample buffer and loaded on SDS-PAGE gels. The presence of CDCP1 and caveolin-1 was analyzed by Western blot. Bands at the predicted molecular weight for CDCP1 (140 kDa) and caveolin-1 (22 kDa) were observed. The figure shows representative gels of 2–4 experiments.
In order to investigate whether normal prostate epithelial cells release vesicles containing CDCP1, the RWPE-1 cell line was used. As shown in Fig. 3A, the amount of CDCP1 was significantly lower in RWPE-1 cell-released microvesicles than in microvesicles released from PC-3 cells when equal amounts of vesicles were loaded. Furthermore, when the levels of CDCP1 in microvesicles released from the nonmetastatic prostate cancer cell line LNCaP and from PC-3 were compared, again the amount of CDCP1 was significantly higher in microvesicles released by PC-3 cells (Fig. 3C). Based on these results, the detection of CDCP1 in microvesicles isolated from biological fluids \( i.e. \) urine, serum, prostatic fluid, seminal fluid, appears to be an interesting method for detection of metastatic prostate cancer. Isolation of microvesicles released from epithelial cells from biological fluids can be performed with beads coupled to an antibody to epithelial cell adhesion molecule (EpCAM) (11). This molecule is expressed exclusively in epithelia and in epithelial-derived cancer types, and increased levels of EpCAM have been associated with the early development of prostatic adenocarcinoma (50). Because our MS analysis showed that EpCAM is found in microvesicles released by the epithelial prostate cancer line PC-3 (supplemental Figs. S2 and S3), we decided to investigate whether it was possible to immunoloisolate PC-3 cell microvesicles and measure CDCP1 protein levels. As shown in Fig. 3D, EpCAM conjugated dynabeads were able to immunoloisolate PC-3 cell-released vesicles containing CDCP1. The presence of caveolin-1 in the immunoloisolated microvesicles was also determined to confirm the binding of microvesicles to the beads (Fig. 3D).

The presence of the tetraspanin CD151 in PC-3 cell-released exosomes was also verified by Western blot. This protein is interesting because it has been shown to be overexpressed in several cancers, and it seems to induce tumor progression by regulating cell migration through its association with integrins and matrix metalloproteinases (51). Furthermore, it has been reported that CD151 protein expression predicted the clinical outcome of low-grade primary prostate cancer better than histologic grading (52). As shown in Fig. 4A, this protein was enriched in PC-3 cell-released microvesicles compared with lysates. Furthermore, similarly to CDCP1, the levels of this protein were higher in PC-3 cells released microvesicles than in microvesicles released from RWPE-1 cells.

In conclusion, CDCP1 and CD151 emerge as promising prostate cancer biomarkers because both CDCP1 and CD151 are enriched in vesicles released by prostate cancer cells compared with vesicles released by normal epithelial prostate cells, and it has been shown that cancer cells release more vesicles than normal cells (52). In fact, one of these molecules, CD151, has already been shown to be useful as a prostate cancer biomarker in prostate cancer tissue (52). Detection of biomarkers in microvesicles has the advantage of being less invasive. In addition, the general problem of tumor heterogeneity can be avoided.

The third candidate, CD147, also was validated by SDS-PAGE and Western blot. It has recently been suggested that this protein has a potential role as an independent predictor of biochemical recurrence, development of metastasis and reduced overall survival in prostate cancer (53). As shown in Fig. 4B, when equal amounts of protein from PC-3 cell microvesicles and cell lysates were analyzed by SDS-PAGE and Western blot, CD147 was clearly enriched in microvesicles released from PC-3 cells. However, the levels of CD147 in microvesicles from the normal epithelial cell line were not very different from the levels found in microvesicles released by PC-3 cells thus suggesting that analysis of CD147 in microvesicles isolated from body fluids may not be useful to detect prostate cancer.

Finally, other proteins found in PC-3 microvesicles that are known to be associated with or disregulated in prostate cancer are translationally controlled tumor protein (TCTP) and neuroplin. TCTP is an antiapoptotic protein highly expressed in prostate cancer (54) and neuroplin, a receptor for vascular endothelial growth factor (VEGF), has been associated with tumor angiogenesis and migration (55, 56), and it has also been suggested to be a marker for prostate cancer aggressiveness (57). Moreover, it was surprising to find that vinculin, an actin binding protein involved in the interaction between the actin cytoskeleton and the extracellular matrix, was very abundant in PC-3 microvesicles (Table IV). This protein appears as a promising marker because it has recently been reported that vinculin expression is associated with increased tumor cell proliferation and progression in advanced prostate cancer (58). Many other proteins found in PC-3 microvesicles are known to be involved in several cancer diseases \( i.e. \) alpha-enolase, catenins, ephrin type-A receptor, epidermal growth factor receptor, several proteins of the Ras family and...
several CD proteins such as CD4, aminopeptidase-N (CD13), CD81. These proteins require further validation.

Comparison Between the Protein Composition of PC-3 Cell Microvesicles and Microvesicles Derived from other Cancer Cells—During the last years several proteomic studies of microvesicles isolated from cancer cell lines have been performed. We decided to investigate to which extent the proteins identified in PC-3 cell microvesicles overlap with the proteins present in microvesicles released by other cancer cells. A recent study identified 48 proteins with two or more peptides in vesicles released by the human prostate cancer cell line PC346C (59). Rab proteins, Arf proteins, Rho proteins, and guanine nucleotide binding proteins for example were not found in this study and only one integrin family member and one member of the 14–3-3 family of proteins were found. Furthermore, a proteomic analysis of vesicles derived from prostate-cancer metastasis identified 30 proteins (60), only 11 of them were identified in PC-3 cell microvesicles. Annexin isoforms, alpha-enolase, 14–3–3 protein sigma, actin, peroxiredoxin-6, ubiquitin-conjugating enzyme E2N, triosephosphohosphate isomerase, phosphatidylethanolamide-binding protein, and heat shock protein beta-1 were found in the two vesicle populations. Also in that study important components of microvesicles from PC-3 cells such as integrins, Rab proteins, or tetraspanins were not found. Moreover, during the revision of this work an article including a list of proteins from microvesicles released from PC-3 cells was published (61). In our opinion, the two studies complement each other because only 94 of the 266 proteins identified in our study were described by Inder K. L. et al. (61). This may be because of the different experimental approaches used in the two studies.

Finally, the overlap between the 266 proteins identified in PC-3 microvesicles and the 353 proteins identified in vesicles released by a bladder cancer cell line (21) and the 394 proteins identified in vesicles released by a colon cancer cell line (9) was investigated. Approximately 35% of the proteins in PC-3 cell-derived microvesicles were found in bladder cancer cells and in colon cancer microvesicles. In general, these vesicle populations did not overlap to a high extent. This is probably not surprising because it has been shown that microvesicles have a set of proteins specific to the cell line from where they originate. Although, some differences may also be explained by the different methods used for the isolation of the vesicles and/or the different proteomic approach that were used in these studies. The fact the composition of microvesicles is cell-type dependent is important to ascribe their presence in biological fluids to a certain type of cancer.

CONCLUSIONS

Prostate cancer research is facing several challenges, and to find biomarkers for detection, diagnosis, and prognosis is one of them. Microvesicles have recently appeared as a source of cancer biomarkers. We have presented here the results of a proteomic analysis of microvesicles released by the metastatic prostate cancer cell line PC-3. 266 proteins were identified with two or more peptide sequences. Many of these proteins have previously been identified in exosomes thus indicating that PC-3 vesicles have basic features in common with other types of exosomes at the protein level. Importantly, we have identified several proteins that seem to be specific to microvesicles released from prostate cancer cells and which potentially could be useful as biomarkers. These proteins are interesting candidates for clinical validation studies.

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This article contains supplemental Figs. S1 to S4.

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