Published in final edited form as:

Biochim Biophys Acta. 2013 April ; 1834(4): 791–797. doi:10.1016/j.bbapap.2013.01.011.

Subcellular Fractionation Enhances Proteome Coverage of Pancreatic Duct Cells

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

JP, AG, and AG carried out the experiments. JP drafted the original manuscript. JP conceived of the study. JP, PB, DC, and HS participated in its design and coordination. All authors assisted drafting the manuscript and approved the final manuscript.

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Abstract

Objectives—Subcellular fractionation of whole cell lysates offers a means of simplifying protein mixtures, potentially permitting greater depth of proteomic analysis. Here we compare proteins identified from pancreatic duct cells (PaDC) following organelle enrichment to those identified from PaDC whole cell lysates to determine if the additional procedures of subcellular fractionation increases proteome coverage.

Methods—We used differential centrifugation to enrich for nuclear, mitochondrial, membrane, and cytosolic proteins. We then compared - via mass spectrometry-based analysis - the number of proteins identified from these four fractions with four biological replicates of PaDC whole cell lysates.

Results—We identified similar numbers of proteins among all samples investigated. In total, 1658 non-redundant proteins were identified in the replicate samples, while 2196 were identified in the subcellular fractionation samples, corresponding to a 30% increase. Additionally, we noted that each organelle fraction was in fact enriched with proteins specific to the targeted organelle.

Conclusions—Subcellular fractionation of PaDC resulted in greater proteome coverage compared to PaDC whole cell lysate analysis. Although more labor intensive and time consuming, subcellular fractionation provides greater proteome coverage, and enriches for compartmentalized sub-populations of proteins. Application of this subcellular fractionation strategy allows for a greater depth of proteomic analysis and thus a better understanding of the cellular mechanisms of pancreatic disease.

Keywords

organelle enrichment; chronic pancreatitis; pancreatic cancer

1. INTRODUCTION

The development and/or progression of chronic pancreatitis is associated with the dysregulation of cellular processes in the three cell types (acinar, duct and stellate) comprising the exocrine pancreas result in [1]. Further investigation is necessary at the cellular and subcellular level to provide a comprehensive analysis of cellular events under various forms of stress, such as alcohol and tobacco consumption. Human pancreatic duct cells (PaDC) secrete inflammatory mediators and extracellular matrix proteins that have major roles in inflammation and fibrosis, respectively [2]. These secreted proteins have been implicated in pancreatic stellate cell activation and are key to forming the basis of both the primary duct [3] and the sentinel acute pancreatitis event (SAPE) [4, 5] hypotheses of chronic pancreatitis pathogenesis. Currently, such cellular mechanisms have not been resolved comprehensively. A better understanding of the pancreas at the subcellular level will enhance our understanding of the pathophysiological mechanisms regulating pancreatic disease.

Subcellular fractionation using differential centrifugation allows for the separation of organelles based upon their physical properties, thereby reducing protein complexity. Subcellular fractionation of organelles is challenging as contamination of a particular organelle preparation by other organelles may occur at many points during the fractionation protocol. The purity of the fractions is dependent on the rigor of cellular homogenization and the physical characteristics of fractionation necessary to separate the homogenate into the designated populations of organelles [6]. Differential centrifugation is a robust method

for subcellular fractionation, which can be readily applied to PaDC studies investigating pancreatic disease.

Comprehensive proteomic analyses aim to identify and characterize all proteins from a specific cell type or tissue, in all possible states [7]. Subcellular fractionation in tandem with state-of-the-art mass spectrometry-based proteomics represents a powerful tool for expanding the depth of cellular proteome coverage. Subcellular fractionation allows dissection of intracellular organelles, including the isolation of multi-protein complexes from these organelles. As such, many low abundance proteins and a variety of signaling complexes can be enriched, whereas unfractionated whole-cell lysate analyses are dominated by the most abundant proteins.

We use a readily amenable subcellular fractionation strategy to generate enriched fractions of specific organelles for mass spectrometry analysis. Here we fractionate whole cell lysates of PaDC and determine if enriching for proteins categorized as cytosolic, nuclear, mitochondrial, or plasma membrane-associated results in greater proteome coverage. We compare the number of proteins isolated using this subcellular fractionation strategy with that consisting of four biological replicates of unfractionated PaDC whole cell lysates. We observed a greater number of proteins, as well as enrichment of organelle-specific proteins in the intended fractions, using the subcellular fractionation strategy. The method described in this study is well suited for the subcellular fractionation of PaDC and its use will be beneficial to future comprehensive proteomic investigations of PaDC, and other pancreatic cell types, under various forms of cellular stress.

2 MATERIALS AND METHODS

2.1 Materials

Dulbecco's modified Eagle's-F12 medium (DMEM/F12; 11330) was purchased from Gibco (Carlsbad, CA). Fetal bovine serum (FBS; F0392) was purchased from Sigma (St. Louis, MO). CellStripper (25-056-CL) was purchased from Mediatech (Manassas, VA). SeeBluePlus2 Pre-Stained standard (LC5925), LDS (lithium dodecyl sulfate) sample buffer (NP0008), NuPAGE 4–12% Bis-Tris polyacrylamide gels (NP0335), SimplyBlue Coomassie stain (LC0665), and MES-SDS (2-(N-morpholino)ethanesulfonic acid-sodium dodecyl sulfate) electrophoresis buffer (NP002) were from Invitrogen (Carlsbad, CA). Sequencing-grade modified trypsin (V5111) was obtained from Promega (Madison, WI). Other reagents and solvents were from Sigma-Aldrich and Burdick & Jackson, respectively. The PaDC cell line, hTERT-HPNE (CRL-4023), was purchased from ATCC (Manassas, VA).

2.2 Experimental Workflow

The experimental workflow for our differential centrifugation strategy, summarized in Figure 1, was as follows: A) PaDC were grown in DMEM/10%FBS in 5 cell culture dishes. The cells were harvested. Whole cell lysates from 4 dishes were used as biological replicates. Cells from the remaining cell culture dish were lysed and subjected to differential centrifugation to isolate B) nuclei, C) mitochondria, D) cell membranes, and E) cytosolic proteins.

Proteins in the differential centrifugation fractions and the biological replicates were separated by SDS-PAGE and processed using standard GeLC-MS/MS techniques. Finally, the collected mass spectrometric data were analyzed using ProteomeDiscoverer for protein identification and GoFact for gene ontology classification [8].

2.3 Cell growth and harvesting of pancreatic duct cells (PaDC)

Cell growth and propagation was performed according to previously described methods [9, 10]. The PaDC cell line, hTERT-HPNE (CRL-4023), was purchased from ATCC (Manassas, VA). These cells had been immortalized with transduction with catalytic subunit of human telomerase (hTERT) [11]. In brief, an immortalized PaDC line was propagated in DMEM supplemented with 10% FBS. Upon achieving 90% confluency on 15 cm cell culture dishes, the growth media was aspirated and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS). The cells were dislodged with non-enzymatic CellStripper, harvested by trituration, and pelleted by centrifugation at $3,000 \times g$ for 5 min at 4°C. The supernatant was then removed and the cells washed again with PBS.

2.4 Cell lysis and subcellular fractionation

Differential centrifugation procedures were based upon a previously developed methods [12]. Five hundred microliters of fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1mM DTT (dithiothreitol) and 1X Roche Complete protease inhibitor cocktail) was added to the cell pellet marked for differential fractionation. Cells were homogenized by 10 passages through a 25 G needle using a 1 ml syringe and incubated on ice for 30 min.

The nuclear pellet was extracted by centrifugation at $720 \times g$ for 5 min. The supernatant (S1) was deposited into a new microcentrifuge tube. The nuclear pellet was washed once by adding 500 μ l of fractionation buffer, dispersed with a pipette, passed through a 25 G needle 10 times and centrifuged again at $720 \times g$ for 5 min. The wash buffer was removed and the nuclear pellet was resuspended in Laemmli buffer, sonicated briefly (3 sec) on ice, and stored at -20°C until analysis.

The supernatant (S1) was centrifuged again at $10,000 \times g$. The resulting supernatant (S2) was deposited into a new microcentrifuge tube. The pellet (mitochondrial fraction) was washed once by adding 500 μ l of fractionation buffer, dispersed with a pipette, passed through a 25 G needle 10 times and centrifuged again at $10,000 \times g$ for 10 min. The wash buffer was removed and the mitochondrial pellet was resuspended in Laemmli buffer, sonicated briefly (3 sec) on ice, and stored at -20°C until analysis.

The supernatant (S2) was centrifuged at $100,000 \times g$ for 1 hr. The resulting supernatant (cytosolic protein fraction) was deposited into a new microcentrifuge tube. The pellet (membrane fraction) was washed once by adding 500 μ l of fractionation buffer, dispersed with a pipette, passed through a 25 G needle 10 times and centrifuged again at $100,000 \times g$ for 1 hr. The wash buffer was removed and the membrane protein pellet was resuspended in Laemmli buffer, sonicated briefly (3 sec) on ice, and stored at -20°C until analysis.

Protein concentrations were determined using the bicinchoninic acid (BCA) assay (23225, ThermoFisher Scientific) for the soluble protein fraction and via densitometry estimates for the remaining fractions. The soluble protein fraction was resuspended in Laemmli buffer. Approximately 100 μ g of protein were analyzed via SDS-PAGE.

2.5 SDS-PAGE analysis

A final concentration of 50mM DTT was added to each sample which was incubated at 56°C for 1 hour. After cooling, samples were alkylated with 1% acrylamide for 30 minutes at 23°C. Proteins (approximately 100 μ g) were fractionated by SDS-PAGE at 150 volts in MES (2-(N -morpholino) ethanesulfonic acid) buffer for 45 minutes. Gels were rinsed in deionized water for 10 minutes, fixed in 45% methanol/45% water/10% acetic acid for 30

minutes, stained with SimplyBlue Coomassie for 1 hour, and destained overnight in deionized water.

2.6 GeLC-MS/MS analysis

In brief, each gel lane was divided into 6 sections and proteins in each gel section were digested with trypsin [13, 14]. Peptides extracted from each gel section were fractionated by a nanoflow reversed-phase ultra-high pressure liquid chromatography system (nanoLC, Eksigent) in-line with a linear trap quadrupole-Fourier transform ion cyclotron mass spectrometer (LTQ-FT Ultra, Thermo Scientific). The reversed-phase liquid chromatography columns (15 cm × 100 µm ID, New Objective, Woburn, MA) were packed in-house (Magic C18, 5 µm, 100 Å, Michrom BioResources). Samples were analyzed with a 60-minute linear gradient (5–35% acetonitrile with 0.2% formic acid) and data were acquired in a data-dependent manner, with 6 MS/MS scans for every full scan spectrum.

2.7 Database searching

All data generated from the gel sections were searched against the IPI-human database (v3.69) using both the Mascot and SEQUEST nodes and results were combined with Percolator [15] all of which was processed using ProteomeDiscoverer (v 1.3; ThermoFisher Scientific). Two miscleavages were allowed per peptide and mass tolerances of ± 10 ppm for precursor and of ± 0.8 Da for fragment ions were used. Amino acid modifications: fixed: propionamide (Cys); variable: deamidation (Asn/Gln) and oxidation (Met). We allowed onto our lists proteins identified by a single peptide [16, 17]. Our false discovery rate (FDR) of 1% at the protein level was determined by searching the same dataset against the target database and a decoy database; the latter featured the reversed amino acid sequences of all the entries in the IPI-human database (v3.69) [18, 19]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony, thus the proteins we present represent those with the highest scores for that particular protein group.

2.8 Venn diagrams

The VENNY on-line Venn diagram plotter was used to obtain lists of proteins exclusive to or in common among the sample types investigated [20].

2.9 Gene ontology analysis

Gene ontology analysis [21] was performed manually with the UniProt [22] database or using the GoFact online tool [8, 23].

3 RESULTS

3.1 GeLC-MS/MS analysis revealed distinct banding patterns for organelle-enriched fractions

The four biological replicate samples showed similar protein banding patterns, as was expected (Figure 2, **left**). Conversely, the four subcellularly-enriched fractions showed relatively different overall protein banding patterns (Figure 2, **right**). Of note were the histone proteins which were visualized in the 14–17 kDa region on the SDS-PAGE image in the nuclear protein lane, an abundance of these proteins was expected in the nuclear-enriched fractions. As such, it followed that mass spectrometry-based proteomic analysis would likely identify a wider range of proteins among these organelle-enriched samples than would be identified in the biological replicate samples.

3.2 Similar numbers of proteins were identified among the eight samples investigated

We performed GeLC-MS/MS analysis of all 8 sample lanes fractionated into 6 fractions each to compare the proteins between and within the subcellular fractionation and biological replicate groups. This mass spectrometry-based proteomic analysis identified several hundred proteins in each of the 8 lanes. On average, 928 proteins were identified among the biological replicates, while an average of 918 proteins were identified for the subcellular fractionation samples (Table 1). A Student t-test showed no evidence of statistically significant difference (p -value= 0.909) between these two sets of samples. In total, we identified 1658 non-redundant proteins in the biological replicate group, while we identified 2196 non-redundant proteins in the subcellular fractionation group. These values correspond to an increase of approximately 30% more protein identifications when subcellular fractionation was performed compared to PaDC whole cell lysates.

3.3 Differences were observed in the protein overlap within biological replicates and subcellular enriched fractions

We next examined the overlap of proteins among the four subcellular fractions and among the four biological replicates of PaDC whole cell lysates. Venn diagrams were used to illustrate the overlap among the four samples within each group, i.e., biological replicates (Figure 3A) and subcellular fractions (Figure 3B). Figure 3C summarized the number of fractions in which proteins were identified. In the biological replicate group, approximately 22% of the total proteins were identified in all 4 samples, as opposed to the organelle fractionation group in which only approximately 6% of the total proteins were identified in all four organelle-enriched samples. As expected, the overlap among biological replicates was high compared to that of the subcellular-fractionated samples. Conversely, when examining the number of proteins that were exclusive to a particular group, 39% of the proteins in the biological replicate samples appeared in only a single replicate, while nearly 64% of the proteins in the organelle fractionation samples appeared in only one fraction. We have shown that subcellular fractionation enriched for a sub-population of proteins that were not identified in the biological replicate samples. Figure 3D illustrates that approximately twice as many unique proteins were identified in the amalgamated organelle-enriched samples. We suspect that with greater number of replicates in the organelle-enriched samples, proteins those proteins exclusively in the Rep0–3 group will decrease as the proteome coverage of the organelle-enriched samples increases. This result indicated that subcellular fractionation of PaDC allowed for a greater depth of proteome analysis than the biological replicates, which was a critical factor for investigating proteins of lower abundance.

3.4 Gene ontology analysis of proteins exclusive to the subcellular fractions revealed expected enrichment of organelle-specific proteins

To illustrate that unique sub-proteomes were isolated and that these sub-proteomes were indeed specific to the intended organelle of interest, we analyzed those proteins exclusive to each fraction via gene ontology classification [8]. The classification categories were limited to cytosol, mitochondria, nucleus, and plasma membrane. The results of the gene ontology classification (using GoFact) showed that the enrichment did not result in fractions being homogeneous to the targeted organelle, as cross-contamination was observed amongst the four organelle-enriched fractions (Figure 4). However, the major category identified for each fraction was indeed the intended category. That is, proteins identified exclusively in the nuclear fraction were classified primarily as being of nuclear origin, those exclusive in the mitochondrial fraction were classified primarily as mitochondrial in origin, and similarly for membrane and cytosolic proteins. Of note were the nuclear proteins which appeared as the second most abundant in the non-nuclear protein fractions. In Figure 4, we also note the gene ontology classifications of proteins that were identified in all 4 replicate cell lysate

samples (RepCore). As expected, the distributions of these proteins in cell lysate looked much like the cytosol fraction, as no enrichment has been performed. Unintended lysis of nuclei during homogenization could have likely led to this scenario, and as such future studies should use less harsh homogenization conditions (e.g., fewer passages through the syringe or using a dounce homogenizer in lieu of syringe lysis) and/or ensure that nuclei remain intact using light microscopy. Although we were able to identify a more diverse proteome with subcellular fractionation, cross-contamination from other organelles was prevalent. Such a finding indicated that additional optimization of lysis and fractionation conditions are warranted, and successful implementation of such will likely expand further the depth of proteomic analysis.

DISCUSSION

Our data have demonstrated the advantages of subcellular fractionation of PaDC in the enhancement of the number of proteins identified by mass spectrometry analysis. Performing subcellular fractionation, although it added approximately 4-hrs to the protocol, increased the number of proteins identified by approximately 30%. We identified 2196 proteins, which included 538 more proteins than that which was observed in the analysis of PaDC whole cell lysate. As expected, many proteins (approximately 64%) that were identified in the organelle-enriched samples were identified exclusively in that particular organelle fraction. Although our protocol requires further optimization to reduce cross-contamination from other organelles and to achieve a purer organelle preparation, the main gene ontology category for each organelle-enriched fraction is that which is expected. Therefore, subcellular fractionation is indeed a valuable procedure which enables an increased depth of proteomic analysis.

A significant advantage of subcellular fractionation is that the added dimension of separation can be attained as proteins regulating the functions of select individual organelles can be probed more in depth. Techniques of subcellular fractionation often exploit various physical parameters (i.e., size, density or charge) or biological properties (i.e., immunoaffinity) [24]. In addition to differential centrifugation, other methods of subcellular fractionation include free-flow electrophoresis[25], differential density ultracentrifugation[6], or differential detergent centrifugation (DDC) [26]. More recently developed methods also include immunoaffinity purification [27] and fluorescent-assisted organelle sorting (FAOS) [28, 29]. Compared to differential centrifugation, these techniques have certain advantages related to enhanced organelle purity, however, these alternative fractionation strategies do have inherent caveats. Free-flow electrophoresis and FAOS, for example, require specialized equipment. Immunoaffinity purification, as well as certain modalities of FAOS, necessitate surface accessible marker proteins on organelles to enable their isolation. Of these alternatives, DDC, which uses detergents and avoids long centrifugations, has shown promise for comprehensive subcellular proteomic analyses[26, 27, 30], but cross-contamination remains a major limitation and detergent removal is a prerequisite for mass spectrometric analysis..

We chose to employ differential centrifugation as it is a technically simple and readily applicable method of subcellular fractionation requiring only a commonly accessible and typically ubiquitous ultracentrifuge. Differential centrifugation has the advantages of not requiring additional specialized equipment and not relying on the quality of antibodies; however these advantages come at the expense of poor resolving power that can result in a greater degree of cross-contamination of different organelle types. Cross-contamination of organelles for which the enrichment is not directed is a common caveat of this subcellular fractionation method [6]. Such cross-contamination is present in our own work (Figure 4). As an alternative quality control measure, quantitative western blotting or immuno-

fluorescence can be used to assess the enrichment of organelles by using organelle marker-specific antibodies or electron microscopy, if available [31]. The use of such validation methods would be valuable for future studies aimed to optimize further our subcellular fractionation strategy.

In the study described herein, we have presented a two-dimensional fractionation strategy at the protein level for the analysis of the PaDC proteome. Four distinct specific organelle-enriched fractions were collected and the extracted proteins were subjected to an additional dimension of fractionation via SDS-PAGE. Similar techniques may be applied to other cell lines, including HEK, HeLa and CHO, for which similar results may be expected. To increase further the depth of proteomic analysis at the expense of increased duration of sample preparation, additional fractionation methodologies may be necessary. Such procedures can be performed at the protein level (i.e., using two-dimensional gels in lieu of one-dimensional SDS-PAGE) and/or at the peptide level (i.e., OFFGEL fractionation or strong cation exchange). In fact, strategies have been developed for over 20 subcellular compartments [32]. The greater resolving power obtained by further fractionation will lead to less complex protein mixtures and thus increase the sensitivity of the ensuing proteomic analysis.

CONCLUSIONS

We have presented a platform which can be applied to studying perturbations in select organelles of PaDC under various conditions. The development of chronic pancreatitis and the molecular mechanisms regulating dysfunction of the exocrine pancreas at the cellular level remain unresolved, although several risk factors have been implicated. The strategy which we present herein can be applied to the investigation of select risk factors. For example, the proteomic changes occurring as a result of cellular stress - such as that produced from incubation of the cells with ethanol, cytokines, nicotine, or cigarette smoke carcinogens - can be determined at the organelle level using the protocol outlined herein. An example of the utility of our methodology would be in the study of mitochondrial autophagy and its role in chronic pancreatitis and pancreatic cancer [33, 34]. Also with highly pure organelle fractionation, proteins that traffic between different cellular compartments can be followed with time course-based experiments. Such analyses would be valuable in elucidating pathways which are attenuated during cellular stress in the context of the development of pancreatic disease. Through the work presented herein, we have laid the foundation upon which further studies of the subcellular proteome of PaDC may be developed. The combination of subcellular fractionation and mass spectrometry allows for high-throughput protein identification, yet provides greater proteomic depth, as sub-populations of proteins are identified at the organelle level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funds were provided by the following NIH grants: 1 F32 DK085835-01A1 (JP), 1 R21 DK081703-01A2 (DC) and 5 P30 DK034854-24 (Harvard Digestive Diseases Center; DC), as well as a grant from the American College of Gastroenterology: ACG – 042103580 (JP). In addition, we would like to thank the Burrill family for their generous support through the Burrill Research Grant. We would also like to thank members of the Steen Lab at Children's Hospital Boston, in particular Scott Brizard, and John FK Sauld, as well as Shadeah Suleiman (Research Manager) from the Center for Pancreatic Disease at Brigham and Women's Hospital for their technical assistance and critical reading of the manuscript.

LIST OF ABBREVIATIONS

DMEM	Eagle's minimal essential medium
FBS	fetal bovine serum
GeLC-MS/MS	SDS-PAGE (gel) coupled with liquid chromatography tandem mass spectrometry
hPaDC	human pancreatic duct cells
PaDC	pancreatic duct cells

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HIGHLIGHTS

- SDS-PAGE revealed distinct banding pattern for organelle-enriched fractions
- Similar numbers of proteins were identified by each method
- Greater overlap of proteins within replicates than within subcellular fractions
- Gene ontology revealed expected enrichment of organelle-specific proteins

Figure 1. Experimental workflow

The experimental workflow was as follows: A) PaDC were grown in DMEM/10%FBS in 5 cell culture dishes. The cells were harvested, and whole cell lysates from 4 plates were used as biological replicates. Cells from the remaining culture dish were lysed and subjected to differential centrifugation to isolate B) nuclei from the first pellet, C) mitochondria from supernatant S1, D) cell membranes from the second pellet, and E) cytosolic proteins (supernatant S2).

Figure 2. SDS-PAGE analysis

Three replicate lanes of whole cell lysate from PaDC were fractionated via SDS-PAGE (left). In addition, the proteins from subcellular fractionation samples were separated further by SDS-PAGE (right). Rep, replicate.

Figure 3. Comparing protein identifications for each set of samples

A) Venn diagram comparing four biological replicates of PaDC whole cell lysates. B) Venn diagram comparing the four subcellular fractions of PaDC. C) Comparison of the redundancy of proteins identified among replicates and among organelle fractions. Proteins were tallied which appeared in 1 (horizontal lines), 2 (vertical lines), 3 (diagonal lines), or 4 (dots) fractions/replicates. D) Venn diagram comparing all proteins identified in all four biological replicates of PaDC whole cell lysates with those in all four organelles samples.

Figure 4. Gene ontology analysis of proteins in each fraction

The proteins identified exclusively in each organelle-enriched fraction and those proteins identified in all 4 replicate samples (RepCore) were subjected to GOfact for gene ontology analysis.

Table 1

Summary of proteins identified.

sample type	individual sample	proteins identified in each sample	non-redundant proteins identified in each sample type
biological replicate	Rep0	812	1658
	Rep1	1004	
	Rep2	905	
	Rep3	989	
subcellular fractionation	cytosol	817	2196
	nucleus	1000	
	mitochondria	788	
	membrane	1066	