Subcellular proteomics fractionation in neutrophils –enabling signaling transduction studies–

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Introduction

Sample preparation is a crucial stage in proteomics analysis. Proper experimental model and careful sample preparation is vital to obtain significant and trustworthy results. In the past years, efforts in reducing dynamic range of the proteins and enhancing detectability have been a major focus of several proteomics studies. One of the major hurdles associated with proteomic analysis of such complex sample is the high dynamic range of proteins (1). Since most of the regulatory proteins such as kinases and GTPases are present in low copy numbers, important layers of information are missing from studies of whole cell proteomics. Combining large-scale proteomics approaches with traditional cell-biology techniques is providing a strategy for mapping proteins in cell compartments (2), known as Subcellular Proteomics. From a technical perspective, it has also provided an interesting means to reduce complexity of cellular proteome and partially overcome the abovementioned resolution limitation of proteomic separation technologies (3). Recently, two groups have applied a subcellular fractionation approach to characterize proteins of neutrophil granules (4) and phagosomes (5) leading to the identification of 286 proteins on the three granule subsets and more than 198 non-redundant phagosome proteins.

Through in-silico analysis, we have observed that most of the proteins involved in signaling transduction during neutrophil migration, extravasation, phagocytosis, degranulation and apoptosis reside in cytosol (12). In the present study, the nonionic detergent ***Digitonin***, which binds specifically and rapidly to sterols and precipitates them out, was used to allow the formation of pores in the membranes and release of soluble cytosolic proteins (6). The use of digitonin at low concentrations (7) and the large differences in sterol contents of the plasma membrane and the cell organelles (8) and other organelles allow selective lysis of the sterol-rich plasma membrane while keeping more or less intact sterol-poor intracellular membranes (9).

Material and methods

Peripheral neutrophils were purified from human blood by centrifugation on Percoll density gradient as described in Campos A. (2007). The degree of purity was evaluated by flow cytometry and found to be at least 98%. Digitonin fractions were obtained by resuspending the cells in 5 volumes of digitonin extraction buffer (0.015% w/v digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM PIPES pH 7.4) containing inhibitors of proteases and phosphatases for 10 min at 4°C with gently shaking. The extracted cytosolic proteins were precipitated overnight with 4 volumes of ice-cold acetone. The pellet was resolubilized in IEF rehydration solution and the protein concentration was determined using the 2D Quant Kit (GE Healthcare) as recommended by the manufacturer. Two hundred μg of cytosolic-fractionated protein was used for 2D-PAGE (IPG strips 3-10 NL 24cm.) as described before (10). After electrophoresis, the slab gels were stained using an amoniacal staining procedure (11). Stained gels were then imaged on an ImageScanner (Amersham Pharmacia) using the LabScan software (v.3). Gel images were imported into the ImageMaster software (v.6) for automated spot detection and analysis.

In-gel digestion was carried out as described by Shevchenko et al. (12). The digests were dried in a vacuum centrifuge and then redissolved in 0.1% TFA. Each digest sample was desalted on a POROS R3 microcolumn, and eluted with CHCA in 50% acetonitrile directly onto the MALDI target plate. Tryptic digests were analyzed by MALDI-TOF/TOF MS (ABI 4700). MS spectra were acquired in positive reflector mode, and the three major peaks were selected for further MS/MS analysis. The resulting MS and MS/MS spectra were collectively used to interrogate sequences present in the Swiss-Prot and NCBInr databases. Searches were performed with complete carbamidomethylation of cysteine, with partial oxidation of methionine residues, and with

one missed cleavage. Mass tolerance was set at 100 ppm for the masses of peptide precursors and at 0.25 Da for the masses of fragment ions.

Results and conclusion

Following cytosolic protein fractionation from neutrophils and separation by 2D-PAGE, approximately 700 spots were detected using amoniacal silver staining. The optimal concentration of 100 μM digitonin was found to affect selectively the cytoplasmic membranes of neutrophils, releasing the cytosolic proteins with little contamination from the organelles or plasma membrane. To confirm the efficiency of our digitonin enrichment method, spots were randomly excised, digested and identified by MALDI-TOF/TOF. To date, over one hundred proteins have been identified as primarily or alternatively cytosolic proteins. To assess the reproducibility of the proposed method for cytosolic proteins enrichment, sample preparation procedure were carried out separately (in different days). To minimize the influence of the biological variability as much as possible, the same sample donor for the three replicates was used. A high degree of reproducibility was reached among the three sample preparations. Using differential gel image analysis software, we found that the three gels were at least 80% similar in terms of spot volume after normalization (Pearson correlation coefficient of 0.899). Importantly, this analysis was performed uniquely with the 'automatic matching' features of the ImageMaster Platinum software, with no manual editing (operator editing) carried out.

The results presented here suggest that our method for cytosolic protein enrichment offers great efficiency and reproducibility. This protocol is robust, rapid, unexpensive and may be well-suited for analyses of other complex specimens. Last but not the least, as the extraction using digitonin neither denature nor alter the conformation of proteins, cytosolic protein complexes would be expected to be recovered, which would enable future studies of protein-protein interactions.

References

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