CHAPTER 3

MICROPARTICLES: A NEW TOOL FOR PLASMA MEMBRANE SUB-CELLULAR PROTEOMIC

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1. INTRODUCTION

Membranes are critical components of cellular structure. It has been reported that plasma membrane proteins represent about 30% of all cellular proteins (Wallin and von Heijne 1998). Even if plasma membrane was considered for a long time as a simple biological barrier between the cytosol of the cell and the extra-cellular environment, these membrane proteins have been demonstrated to play a crucial role in the different fundamental biological processes as exchange of component or signal transduction. Also, more than half of all anticipated pharmacological drug targets are predicted to be localized to the plasma membrane (Jang and Hanash 2003). Plasma membrane can then clearly be considered as a sub-cellular compartment of first interest in regard to different diagnosis and/or therapeutic target proteins. Indeed, for each membrane protein there is potentially a specific antibody which can be used

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for the diagnosis of several pathologies and also for treatment using armed antibodies (Harris 2004). Therefore, proteomic analysis of plasma membrane proteins is of first importance. Despite the importance of plasma membrane proteins, there is less understanding in this class of proteins due to the difficulty to obtain enriched plasma membrane proteins preparation from eukaryotic cells. Until now many different strategies have been applied but are still laborious and imperfect. In example of these different approaches, biotinylation (Peirce et al. 2004; Zhao et al. 2004) silica coated (Rahbar and Fenselau 2004), partition phase repartition (Qoronfleh et al. 2003) or partial tryptic surface digestion have been tested, but they still remain unsatisfactory. Although some studies of plasma membrane proteins using 2-DE have been reported (Galeva and Altermann 2002; Luche et al. 2003), but the separation of such hydrophobic proteins have been often poor (Santoni et al. 2000). We describe here a new strategy in order to increase the proportions of plasma membrane proteins identified with the highest possible coverage percentage in the different membrane proteins preparations analyzed by mass spectrometry. We will focus on microparticles as a source of plasma membrane proteins.

2. MICROPARTICLES

Each of the two leaflets of the plasma membrane bilayer has a specific lipid composition. Aminophospholipids (phosphatidylserine and phosphatidylethanolamine) are specifically segregated in the inner layer of the membrane, whereas the phosphatidylcholine and the sphingomyelin are enriched in the external leaflet (Bevers et al. 1998). When cells are submitted to various stress conditions as mitogenic activations or apoptosis, the constitutive asymmetry between the inner and the outer leaflet of the plasma membrane is disrupted. The major changes in the plasma membrane constitution will be the delocalization of the phosphatidylserine to the outer leaflet and an augmentation of the Ca^{2+} ion concentration in the cytoplasm. Such changes are going to disrupt the organisation of the cytoskeleton and drive to a blebbing of the plasma membrane and release microvesicles which are named microparticles (MPs) (Figure 1) (Hugel et al. 2005; Miguet et al. 2005).

Such microvesicles have size variable between 50 nm to 1 μm and differ from other vesicles (like exosomes (30–100 nm)). In general, microparticles are phospholipids vesicles derived from eukaryotic cells as a result of different types of stimulation. Microparticles can also be defined as phospholipids microvesicles containing certain membrane proteins originating from the parental cell. Microparticles circulate in the blood and contribute to numerous physiological processes. MPs have been described in various haematopoietic cells as platelets (Heijnen et al. 1999), T-cells (Blanchard et al. 2002), polynuclear neutrophils (Mesri and Altieri 1999) or dendritic cells. After have been considered as cell dust, MPs are now considered to reflect cell activation. Platelet derived microparticles have been the most extensively studied until now. They are now accepted to play an important role in the procoagulant

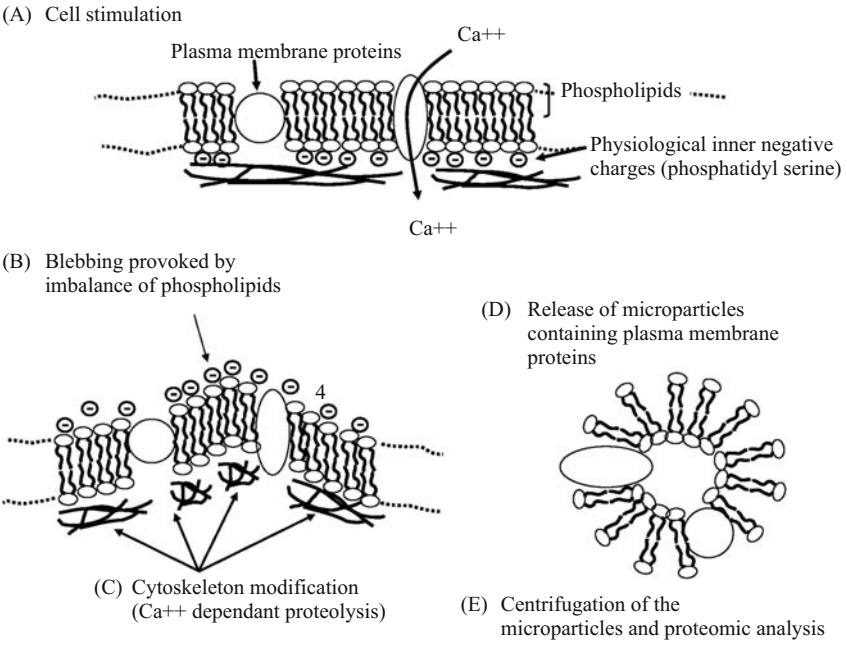

Figure 1. Scheme of microparticles formation. Cell stimulation (A) induces a remodelling of the phosphatidyl serine repartition (B) and an increase of cytosolic calcium concentration (C). Such remodelling induces the MPs formation (D) which are easily separated by centrifugation (E).

process due to the presentation of phospholipids as phosphatidylserine to other platelet which activate them for example (Hugel et al. 2005; Pilzer et al. 2005; Simak and Gelderman 2006).

As microparticles are microvesicles formed directly from plasma membrane, exploration of their protein composition in different pathologies may provide valuable plasma membrane markers specific of the cells they originate from (Pilzer et al. 2005). We then proposed to take the advantage of the physiological production of MPs in order to increase the proportion of plasma membrane proteins and lower the number of irrelevant proteins of the sample.

3. STRATEGY USED FOR MICROPARTICLES PROTEOMIC ANALYSIS

3.1. Presentation of the Strategy

Microparticles are naturally enriched in plasma membrane proteins. They correspond themselves to a sub-proteome, and it is tempting to use them as a substitute to

perform plasma membrane proteomics. The methodology described bellow has been developed to perform the proteomic analysis of microparticles. The major problem is linked to the fact that proteins extracted from microparticles are very hydrophobic and therefore cannot be separated on 2-DE (Santoni et al. 2000). Indeed, as a strong ionic detergent must be used for solubilization of the hydrophobic proteins an electrofocalisation step cannot be performed. The protein extract can then only be separated on 1D-SDS-PAGE gel (Galeva and Altermann 2002). The gel lanes obtained are then cut in consecutive pieces of 2 mm. All these pieces (about 100 for a 20 cm gel) are then digested with trypsin. The tryptic peptides are further extracted and analyzed by MALDI-TOF and ESI-nano-LC-MS-MS.

MALDI spectra obtained on these very complex peptide mixtures (several hundred of peptides are expected in a 2 mm slice of 1D gel) only allowed the identification of the few most abundant proteins using MASCOT (http://www.matrixscience.com/), Profound or ProteinProspector (http://prospector.ucsf.edu/).

The peptide mixtures are then analyzed by ESI-nano-LC-MS/MS. The mass data recorded during the different analysis were processed and converted into MassLynx.pkl (Q-TOFII, Waters) or Esquire.mgf (IonTrap HCT+, Bruker) format in order to be submitted to a protein database via the different search engines. This step allowed usually the identification of about 10 to 20 different proteins in each 2 mm gel slices. This strategy could seem to be time-consuming method (a 20 cm gel generates about 100 slices), but this approach allowed the identification of 400–500 proteins for each microparticles preparation. These 400–500 proteins were mainly identified in the 2 mm gel slices regrouped from 20 to 100 kDa (about 50 slices). In each slice an average of ten proteins was identified. This rather low number is probably due to the fact that the protein extracted from each slice is a sub-proteome. The identification of these proteins using nanoLC-MS/MS was often obtained with a low coverage and few peptides, which was probably due to the fact that only major proteins were detected in each 2 mm gel slice. Since sensitivity and chromatographic resolution are probably one of the key elements to improve both the number of proteins identified and the sequence coverage, we have recently used a LC-Chip chromatographic system (Agilent) coupled to an IonTrap (Bruker) on about 10 slices. In these experiments, the number of protein identified was indeed increased (a factor of 30%). Since MALDI-MS analysis performed on the raw peptide extract was very disappointing from the point of view of protein identification, mainly because of the signal suppression effect, we have developed a new MALDI approach. The suppression effect can only be overcome if the number of peptides present in each fraction is reduced. In order to reduce the complexity of the peptide mixture, a chromatographic fractionation step was performed on the tryptic peptides extract. Figure 2 summarizes the strategy and the different analysis performed on each 2 mm 1D gel tryptic peptide extract.

The column used for this separation was adapted in size to the small quantities of peptides extracted from each slice. It was found that micro-chromatography with 0.3 mm diameter columns yield the best compromise for chromatographic resolution

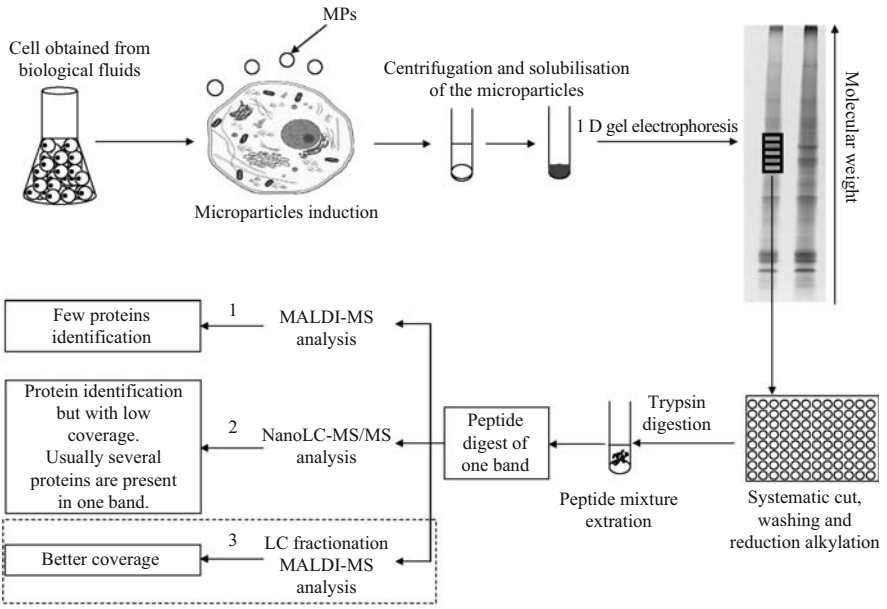

Figure 2. Strategy developed for the microparticles preparation and analysis by mass spectrometry. The step 3 is described in more detail on Figure 3.

and fractionation feasibility. This approach is therefore based on the possibility to collect efficiently fractions of a few μL from a flow rate of about 4 μL/min. In order to speed up the process of data acquisition, a post-column split was used in some cases, allowing to have both ESI-microLC-MS-MS data and fraction collection. Each collected micro-fraction was then submitted to MALDI-MS analysis (0.5 μL used). All MALDI-MS data were than merged to obtain a single peak list showing masses of peptides present in the original extract of one single slice. This single peak list, corresponding to a fictitious MALDI spectrum obtained on the total peptides extract where suppression effect would be absent, allowed to improve very significantly the coverage percentage on the proteins identified by MS-MS data (see bellow).

The "online" or "offline" LC-ESI analysis also generated a list of peptide masses which were usually complementary to the MALDI peaklist. The few microliters left from the collected fractions after MALDI-MS analysis can be used, when necessary, for complementary analysis (MALDI-MS-MS or nano-ESI-MSn).

The complementarity of all the data obtained in this strategy (LC-MALDI-MS and nanoLC-MS/MS) allowed to increase significantly the coverage percentage for all the gel slices. Figure 3 summarizes this strategy and technical details are presented below.

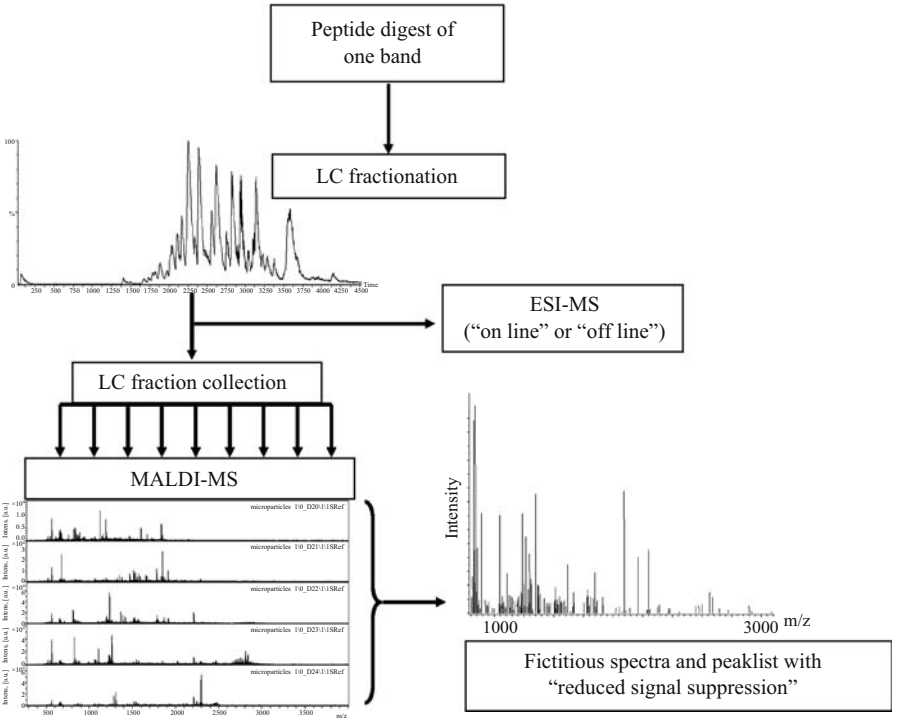

Figure 3. LC-MALDI strategy. Fractionation of the peptide mixture and MALDI-MS analysis.

3.2. Technical Details

The micro-fractionation was realized using a capillary LC system (Agilent 1100 series, Wilmington, DE, USA) equipped with a micro-fraction collector (Agilent 1100 series micro-fraction collector, Wilmington, DE, USA), and all the capillary are optimized for very small column. A total of 15 µl of the samples were injected onto the LC column (Zorbax 300SB-C18, 15 cm × 300 µm, 3.5 µm). Mobile phase solvents were water (A) and acetonitrile (B), containing 0.05 and 0.045% TFA, respectively. Separation was achieved with the following gradient: 5 min at 10% B; from 10 to 80% B in 60 min. The column was maintained at 30°C and the flow rate was set at 4 µL min. Between 3 minutes and 60 minutes, fractions were collected each 2 min in a 96 microwell plate using the automatized micro-fraction collector. Such chromatographic steps generate about 15 fractions containing peptides. All the collected fractions are analyzed by MALDI-MS in order to obtain 20 MALDI-MS spectra. This acquisition is realized on ULTRAFLEX (Bruker) using WARP-LC software (Bruker) for acquisition and treatment of the data (Figure 4).

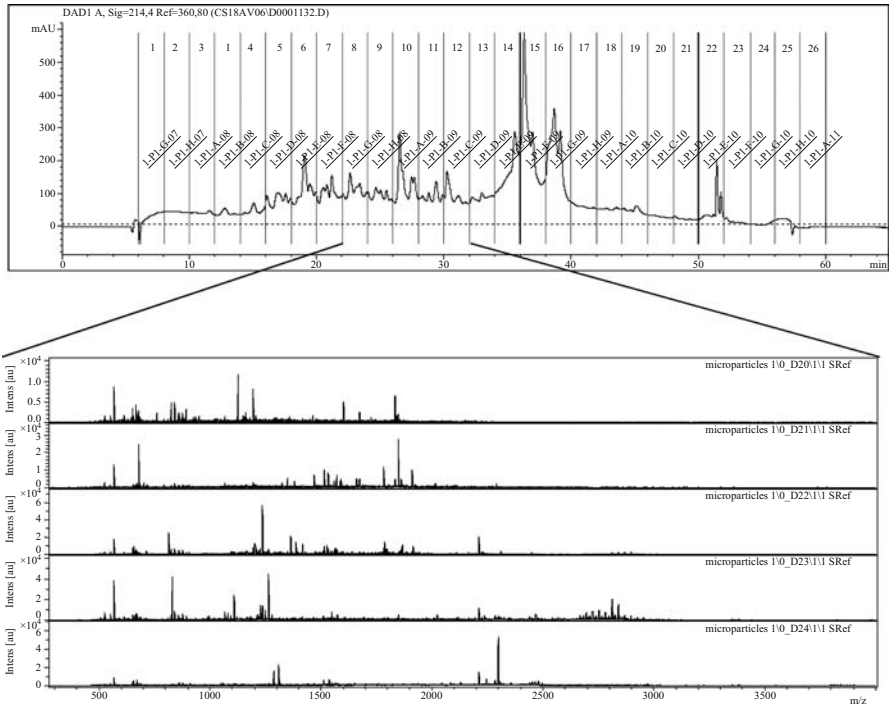

Figure 4. Example of peptide fractionation performed on a 1D gel slice. 26 fractions were collected and analyzed by MALDI-MS. The figure focuses on five of them.

The different spectra are automatically treated in order to generate a single non redundant peaklist. Even if this reconstituted peaklist does not allow new protein identification using peptide mass fingerprint, it combines hundreds of different peptide masses. These masses can be used to obtain a better coverage percentage of the different proteins identified in the different spot. Indeed, in the 1DE LC-MS/MS approach, the identification of the different proteins is performed with few peptides generating low sequence coverage. The different proteins identified by nanoLC-MS/MS are digested *in-silico* in order to generate the theoretical peaklilst. The theoretical and the experimental peaklists are compared together in order to identify the masses corresponding to the tryptic peptides of the identified proteins. The peaks accepted for this compilation must have a signal/noise greater than 5 and a mass error lower than 20 ppm. This strategy allows than a better coverage of the different proteins identified by nanoLC-MS/MS.

Figure 5 summarizes the all strategy, and an example of this approach obtained from the analysis of a single 1D gel slice is reported in Table 1.

Table 1 reports the different proteins identified during the nanoLC-MS/MS analysis of a single 1D gel slice. Peptides obtained from the nanoLC-MS/MS and

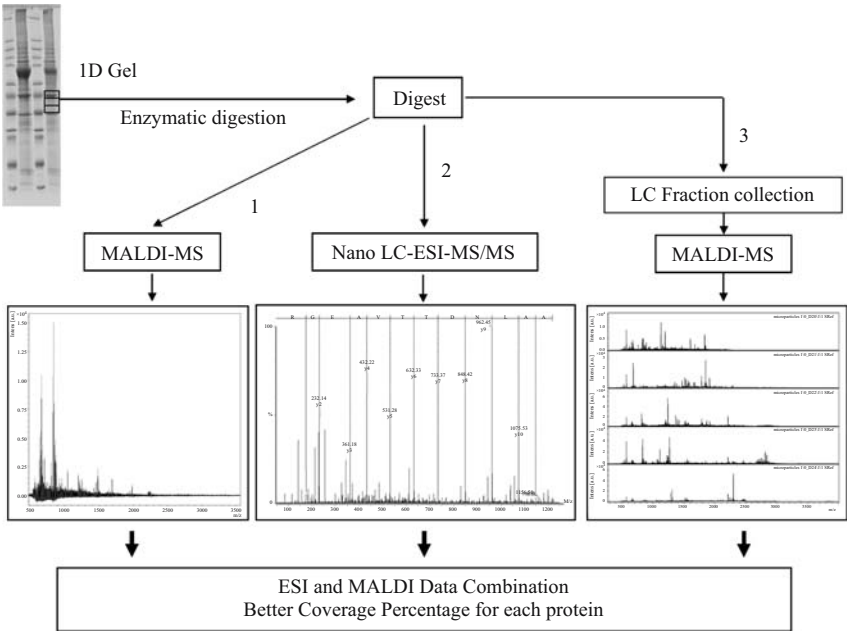

Figure 5. Summary of the strategy applied for a better sequence coverage of the identified proteins in 1D gel. The first step is the analysis of the raw peptide mixture by MALDI. The step 2 corresponds to the nanoLC-MS/MS analysis of the peptide digests, and the step 3 corresponds to the "off-line" fractionation of the peptide digest and MALDI-MS analysis. The combination of these approaches allow a better coverage of the different proteins identified.

LC-MALDI-MS experiments are also reported. The combination of both ESI and MALDI experiments shows well the advantage in term of coverage of this double ionization process. Indeed, some peptides are identified specifically from the ESI ionization process and other from the MALDI process, increasing the whole number of identified peptides.

The improvement of sequence coverage of the proteins is very important first to enhance the confidence of the different identifications and second to have a better characterization of the proteins, particularly in term of post-translational modifications and/or mutations.

Moreover, we have determined the false positive rate for this approach. Many tryptic peptides originated from different proteins can be attributed to a single mass (e.g.: HQHPLQCVMEK 1364.63 Da and EADFINCVIWR 1364.65 Da; $\Delta M < 20$ ppm). Thereby false positive identification may occur. To evaluate the false positive rate, we have selected three common proteins which were not identified during the nanoLC-MS/MS analysis. These proteins (tubulin, actin and myosin) were digested *in-silico*, and the generated mass lists were compared to the LC-MALDI-MS peaklist. A total of only five masses were attributed to the three

Table 1. Summary of the proteins identified by nanoLC-MS/MS from a single 1D gel slice, and the increase of the coverage using LC-MALDI-MS strategy

Accession number	Protein	Peptides identified by nanoLC-MS/MS	Coverage (%)	Peptides identified by LC-MALDI	Combination MALDI/ESI	
					Number of peptides	Final coverage (%)
P08238	Heat shock protein HSP 90-beta	25	31	20	30	47
P07900	Heat shock protein HSP 90-alpha	16	21	27	33	55
P46940	Ras GTPase-activating-like protein	7	5	22	27	22
Q01813	6-phosphofructokinase type C	4	5	7	11	19
P08195	4F2 cell-surface antigen (CD98)	4	9	10	11	30
P23921	Ribonucleoside-diphosphate reductase	3	4	13	14	20
P26639	Threonyl-tRNA synthetase	3	4	13	15	21
P02786	Transferrin receptor (CD71 antigen)	2	2	14	16	29
P16070	CD44 antigen precursor	1	1	3	3	4
Q15758	Neutral amino acid transporter B(0)	1	2	3	4	9

proteins, which is very few compared to the masses attributed to the different proteins in Table 1. This low false positive rate is due to the low tolerance accepted in our strategy (<20 ppm).

To conclude, we have developed a strategy which combines LC-MALDI-MS and nanoLC-MS/MS in order to identify proteins originating from 1D gel with a high coverage compatible with plasma membrane study (CD98, CD71, CD44).

4. VALIDATION OF MICROPARTICLES AS A NEW TOOL FOR PLASMA MEMBRANE PREPARATION

In order to validate the hypothesis that microparticles are structures enriched in plasma membrane proteins, we have analysed the proteome composition of different microparticle preparations obtained from a T-Lymphocytic cell line. Such microparticles can be produced and enriched *in vitro* by mitogenic activation (PHA) or apoptosis induction (Act D1, TNFα) for instance. Microparticles protein mixtures obtained from the two stimulations were separated independently on a 1D gel. The different gels were cut each 2 mm and the different slides were digested and analyzed by nanoLC-MS/MS.

This approach allowed the identification of 390 proteins among which 34% were localized to the plasma membrane. The microparticles obtained from the two different ways of stimulation did not show significant variation in their proteome (Miguet et al. 2005).

These results have been compared to a systematic analysis of a classical plasma membrane protein preparation, generated by ultracentrifugation (100,000 g on sucrose). This plasma membrane isolation has allowed the identification of 292 proteins, and only 69 proteins were located to the plasma membrane (24 vs. 34% for the microparticles preparation). Moreover, 75 plasma membrane proteins were identified specifically in the microparticles preparation, and only 9 were identified only in the plasma membrane preparation. These results are summarized in Figure 6.

On the other hand different groups have started working on the plasma membrane microparticles proteome (Banfi et al. 2005; Garcia et al. 2005) The results obtained by these groups are very different from the results coming from our lab. This might be due to the difference in the microparticles centrifugation step used for MPs isolation. Indeed these two groups used intense centrifugation (between 100,000 and 250,000 g) which is very high in comparison with the theorical microparticles size. This step pellet indeed the microparticles, but also the other vesicles contained in the solution as exosomes which are much smaller. For example, the classical way to prepare exosomes is to centrifuge for half an hour between 100,000 and 200,000 g (Thery et al. 2001; Wubbolts et al. 2003). We believe that this centrifugation step is crucial in order to generate a pure preparation of microparticles. This point was already underlined, even if not discussed, by Jin et al. (2005). In this aim a centrifugation of 20,000 g during 45 min, that we used, is appropriate to generate a homogeneous microparticles preparation (Hugel et al. 2005).

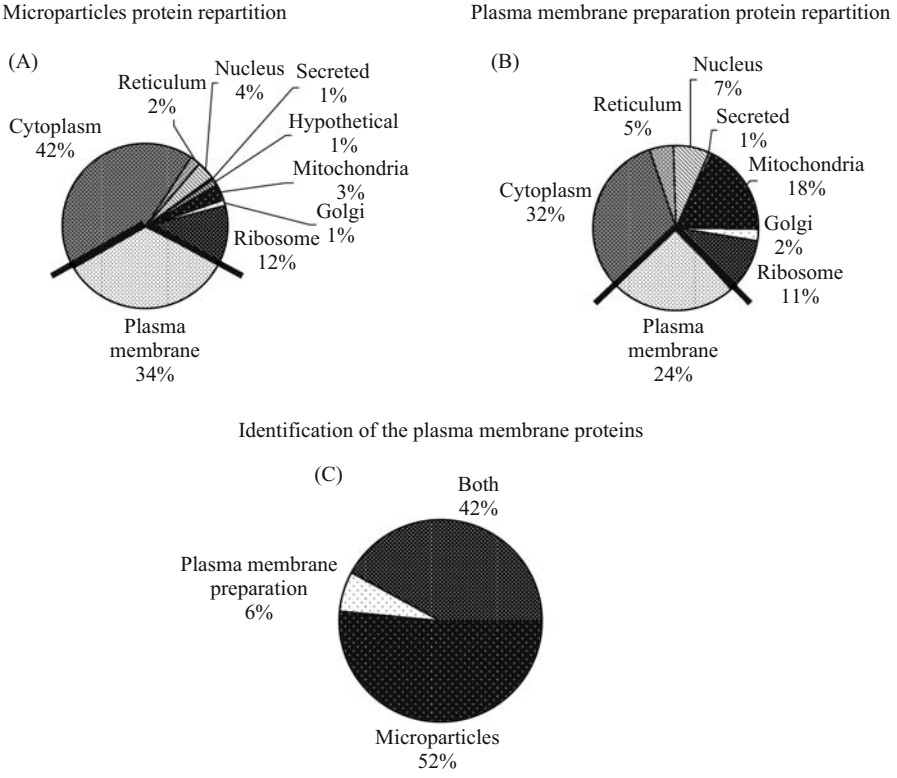

Figure 6. Representation of the protein repartition in function of the preparation. (A) Repartition of the proteins identified in the microparticles. (B) Repartition of the proteins identified in the classical plasma membrane preparation. (C) Comparison of the plasma membrane identified in the microparticles and in the plasma membrane preparation.

5. PERSPECTIVES

As it is potentially possible to generate microparticles from almost all cell types, it could be a new tool for sub-cellular plasma membrane study and biomarker discovery. Even if quantitation is not assessed, the comparison of the microparticles sub-proteome, originating from different pathologies, may enlighten differences in protein expression. As our group is interested in the study of the different B-cell chronic lymphopathies, we have decided to take advantage of the microparticles preparations obtained from various pathologies in order to realize their proteome. In this aim, fresh cells were obtained following informed consent, and subjected to actinomycinD stimulation. The obtained microparticles were first separated on 1D gel before to be systematically analyzed by nanoLC-MS/MS. We analyzed a lymphoma

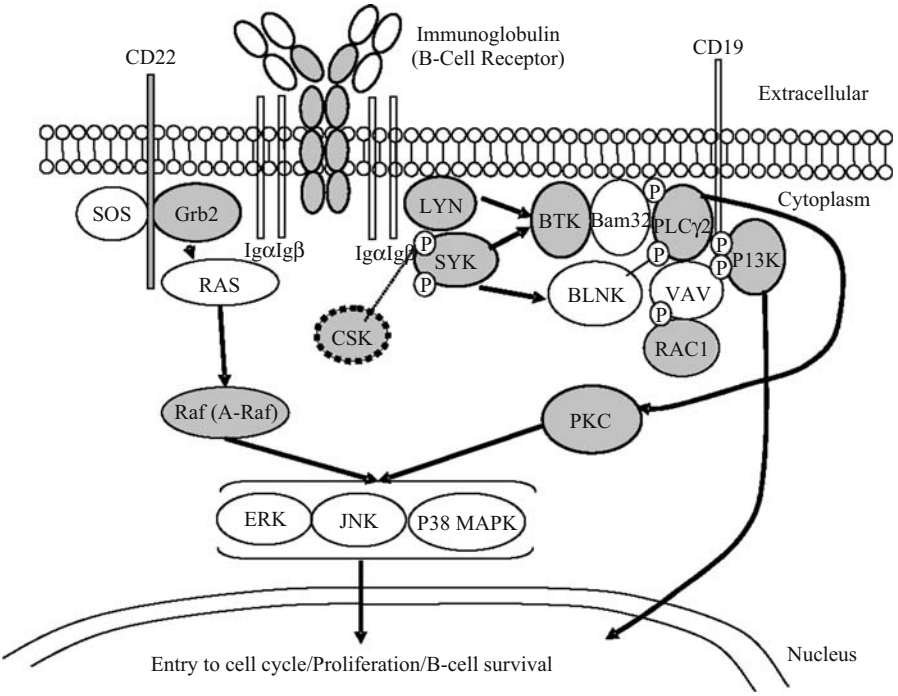

Figure 7. Representation of the B-cell receptor, and the different kinase implied in the signal transduction for the cell proliferation. The proteins in gray were identified in the microparticles obtained from the patient. Most of the proteins implied in the signal transduction were identified using this strategy.

(small cell lymphoma). This analysis has allowed the identification of more than 400 proteins among which 30% were localized to the plasma membrane or associated to the plasma membrane.

Moreover, in this study, a large number of proteins involved in the signal transduction were identified. Indeed B-cell receptor signaling proteins (numerous membrane receptors, kinase proteins) implied in the signal transduction was detected. This complexome identified in the microparticles is reported on Figure 7. The proteins in grey were identified in our study.

In conclusion, plasma membrane microparticles represent a sub-proteome reflecting the plasma membrane composition. These structures have the advantage of not being contaminated by membrane proteins originating from other cell components (golgi, nucleus, mitochondria). As microparticles can be produced by almost all cell lines, they can be useful to discover new potential biomarkers. In this goal we have undertaken the comparison of microparticles originating from different kind of lymphoma. The analytical strategy developed for the characterization of the plasma membrane sub-proteome, based on micro-fractionation, allowed the identification

of hundreds proteins with a better coverage percentage than the classical nanoLC-MS/MS approach. The combinations of nanoLC-MS/MS, micro-fractionation and "off-line" MALDI-MS data is now used routinely in general study for the discovery of new specific biomarkers.

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