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Proteomic Analysis of Membrane-associated Proteins from the Breast Cancer Cell Line MCF7

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Abstract. *Background: Proteins associated with cancer cell membranes represent targets of choice for humoral immune response as well as potential tumour marker proteins in human malignancies. However, proteomic analysis of these proteins, and more generally of low-soluble proteins, remains difficult. Materials and Methods: The breast cancer cell line MCF7 was selected to evaluate a sequential extraction method that enables simple fractionation of human cell proteins according to their subcellular localization, yielding subproteomes enriched in cytosolic and membrane-associated proteins, respectively. A crude plasma membrane preparation was followed by the solubilisation of proteins using trifluoroethanol (TFE) as co-solvent. Results: Cross-matching and statistical analysis performed for each set of two-dimensional electrophoresis (whole-cell, membrane and soluble extracts) and between the different sets highlighted the reproducibility of the extraction process and its usefulness for proteomic analysis. Eighty-three % of the spots of the gels corresponding to the membrane fraction were not found in the gels of the soluble fraction. Conclusion: Due to its simplicity, the approach described here appears well suited for membrane proteomic investigation of human cancer cells and detection of potential biomarkers undetected by current techniques.*

Breast cancer is the second leading cause of cancer deaths in women today (1). In Northern Europe, for the year 2000, the estimated number of deaths from breast cancer was about 21,000 over the 55,000 estimated new cases of breast cancer (2, 3). Even if treatments such as chemotherapy, radiotherapy and anti-oestrogen therapies are still needed, new protein markers have to be identified for early detection. Membrane proteins represent targets of choice for humoral immune response or tumour marker proteins in human malignancies (4-6), and it is clear that the number of candidate membrane proteins involved in carcinogenesis will increase in the future. However, despite their biological importance, proteomic analysis of the proteins associated with the cell membrane remains difficult. They are not yet well recovered in two-dimensional electrophoresis gels (2-DE) (7), widely used to simultaneously study a large number of proteins. In addition to the difficulty of extracting the proteins from cell membranes, their hydrophobic feature renders them poorly soluble in the isoelectric focusing (IEF) step of 2-DE, resulting in severe losses (8, 9). An alternative approach to 2-DE is simple SDS-PAGE, which is much easier to handle and less time-consuming than the 2-D approaches (4, 6, 7, 10). It is powerful enough to estimate the efficiency of extraction and to identify proteins contained in mixtures, but not to monitor dynamic changes or to identify protein isoforms.

Cell membrane proteomics can be improved at different levels: fractionation (sub-proteomics), extraction, electrophoretic separation. The need for enrichment or prefractionation strategies to detect and eventually quantify low-abundance or low-solubility proteins has been addressed previously (11-13). There is a substantial advantage in the proteome analysis which takes into account protein solubility as compared to the global analysis of crude homogenate samples. Many proteins will not show up when crude homogenates are used for the analysis, but will be strongly enriched upon appropriate fractionation (14). Membrane protein separation can be directly performed on a whole cell lysate using a three-step sequential solubilisation protocol (15), or with organic solvent (16). The literature cites a number of procedures for prefractionation, including differential protein solubilisation in increasingly stronger

Abbreviations: GRAVY, grand average of hydrophobicity; TFE, trifluoroethanol; TMRs, transmembrane regions.

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solubilisation cocktails (5, 17), selective removal of dominant protein components (15, 18, 19) and electrophoretic pre-fractionation. Many of those procedures are designed to extract distinct protein fractions from entire cells or tissues. It is likely that the degree of contamination of such protein fractions by other fractions will vary and will not be easily reproducible. Alternatively, an appropriate strategy is to independently map free proteins and membrane-associated proteins. There have been previous attempts to use plasma membrane-enriched preparations from cancer cell lines to discover new marker proteins (4-6). In order to remove soluble protein contaminants, a washing step of the membrane preparation is often added, using salts (13), sodium carbonate (17) or detergent partition (20). Nonetheless, improvements are still needed not only for solubilisation, but also for preparative methods that produce enriched extracts of membrane proteins prior to 2-DE. An elegant sample preparation strategy, based on the use of trifluoroethanol (TFE) organic co-solvent, was applied to *E. coli* membrane proteins (16). Aiming at selectively enriching membrane proteins as well as identifying conditions suitable for 2-DE analysis of human cancer cells, this strategy was adapted to investigate the membrane sub-proteome from the human breast cancer cell line, MCF7. The procedure, allowing separation of both membrane and membrane-associated proteins, consisted of: (i) crude plasma membrane preparation followed by the solubilisation of proteins using TFE as co-solvent; (ii) evaluation of the efficiency of the solubilisation method by SDS-PAGE prior to in-gel digestion and protein identification by nanoLC-MS/MS (ion trap); (iii) evaluation of the usefulness of TFE-induced extraction for 2-DE separation.

Materials and Methods

Materials. The laboratory chemicals were obtained in extra pure grade from Sigma (St. Louis, MO, USA), unless otherwise specified. Carrier ampholyte mixtures (Pharmalytes) and SDS were from GE Amersham Biosciences (Uppsala, Sweden). Linear Immobilines dry strips narrow pH gradient 5-8 (3.3 mm wide and 170 mm long), the molecular weight calibration kit and piperazine diacrylyl (PDA) were from Bio-Rad (Richmond, CA, USA). Agarose low melting and TEMED were from Gibco BRL (Grand Island, NY, USA). Urea was from Merck (Darmstadt, Germany). The protease inhibitor cocktail tablets, Complete (for 50 ml) and Mini-Complete (for 10 ml), were from Roche (Mannheim, Germany).

Preparation of membranes and protein extraction. MCF7 cells, grown to confluence, were washed with 0.9% NaCl and with phosphate-buffered saline (PBS), and scraped using a plastic cell scraper. The cells were centrifuged at 130 x g for 5 min at 4˚C. The cell pellet was washed twice in ice-cold PBS, and resuspended in 4 volumes of lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM orthovanadate and protein inhibitor cocktail) (21). After 15 min at 0˚C, the lysate was homogenized twice with a Dounce glass grinder.

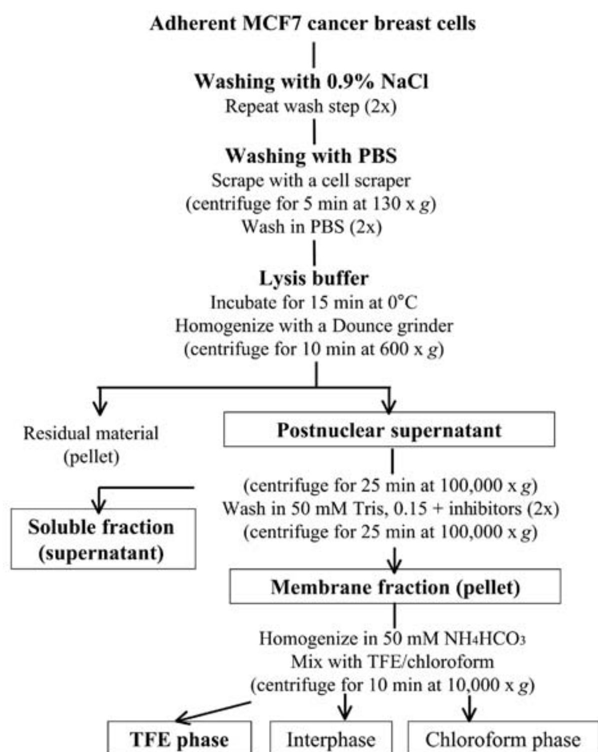

Figure 1. *Schematic representation of the fractionation and extraction procedures. Simplified overview for membrane protein extraction from MCF7 cells.*

Using the method described by Poirier *et al.* (5), the lysate was centrifuged at 600 x g for 10 min at 4˚C, and the postnuclear supernatant was then centrifuged at 100,000 x g for 25 min at 4˚C. The supernatant represented the soluble fraction. The membrane pellet was washed twice in 50 mM Tris, 0.15 mM PMSF and protein inhibitor cocktail, and centrifuged at 100,000 x g for 25 min at 4˚C. The pellet, called the membrane fraction, was extracted using the method described by Deshusses *et al.* (16) for *E. coli* membrane extraction, with minor modifications. This fraction was suspended in 50 mM NH_4HCO_3 (150 µl / 10 mg of dry pellet) and homogenized twice for 3 min with a pellet pestle in a 1.5-ml microtube. A mixture TFE/chloroform (2/1, vol/vol) was added to the microtube (1 ml/150 µl of NH_4HCO_3). The microtube was maintained for 1 h at 0˚C, and mixed quickly with vortex action every 15 min. After a 10-min centrifugation at 10,000 x g at 4˚C, three phases were collected using a syringe: an upper aqueous phase (TFE phase), an insoluble interphase and a chloroformic lower phase. The soluble phases were finally evaporated by vacuum centrifugation.

In addition, for comparative studies, a whole cell extract was prepared in 25 mM Tris, 10 mM EDTA, 7 M urea, 2 M thiourea, 5% v/v glycerol, 0.33% v/v CHAPS, 0.35% v/v Triton X100, 0.35% w/v sulfobetaine 3-10, 10 % v/v isopropanol, 12.5% v/v isobutanol, 100 mM DTT, 1 mM orthovanadate and protein inhibitor cocktail.

Gel electrophoresis. For SDS-PAGE, the dried TFE phase was re-suspended in Laemmli buffer. To evaluate the quality of the extraction method, 5 µg of protein fractions were loaded to 6-cm-long gradient gels (9-18% acrylamide) for SDS-PAGE analyses,

Table I. *Features of the protein identified on 1-D gel in the membrane fraction of MCF7.*

Protein name	SWISS-PROT accession number	Mr gel (kDa)	Protein MW (Da)	GRAVY value	No. of TMRs (TMPred)
60S acidic ribosomal protein P2	P05387	16.1	11665	–0.237	1
Translocon-associated protein	P51571	19.56	18987	0.099	2
40S ribosomal protein S10	P46783	19.56	18886	–0.851	0
ARP2/3 complex 21 kDa subunit	O15145	19.56 22.13	20547	–0.605	0
ARMET protein	P55145	19.56	20256	–0.45	1
Cop-coated vesicle membrane protein p24	Q15363	19.56 22.13 23.68	22761	0.031	2
Transmembrane protein Tmp21 precursor	P49755	22.13	24960	–0.171	2
Peptidyl-prolyl cis-trans isomerase B precursor	P23284	22.13	22785	–0.17	1
60S ribosomal protein L12, mitochondrial	P52815	22.13	21348	0.022	1
Proteasome subunit beta type 2	P49721	22.13 23.68	22822	–0.17	1
Proteasome subunit beta type 6 precursor	P28072	22.13 23.68	25341	0.034	1
Ras-related protein Rab-1B	Q9H0U4	23.68	22157	–0.305	0
Ras-related protein Rap-1A	P10113	23.68	20974	–0.375	1
Peroxiredoxin 2	P32119	23.68	21878	–0.199	1
Ras-related protein Rab-35	Q15286	23.68	23025	–0.473	0
Glycoprotein 25L2 precursor	Q9BVK6	23.68 26.68 27.50	25089	–0.437	2
Ras-related protein Rab-2A	P61019	23.68	23545	–0.354	0
Proteasome subunit beta type 1	P20618	23.68	26489	–0.117	2
Ras-related protein Rab-14	P61106	23.68	23926	–0.411	0
Proteasome subunit beta type 3	P49720	23.68	22949	0.032	0
Ras-related protein Rab-11A	P24410	26.68 27.50	24378	–0.421	1
Ras-related protein Rab-5C	P51148	26.68	23468	–0.333	0
Proteasome subunit alpha type 2	P25787	26.68	25751	–0.195	1
Ras-related protein Rab-6A	P20340	26.68	23578	–0.415	0
Heat shock 27 kDa protein	P04792	26.68 27.50	22768	–0.567	0
Synaptogyrin 2	O43760	26.68	24794	0.168	4
Ras-related protein Rab-5A	P20339	26.68	23658	–0.428	0
Voltage-dependent anion-selective channel	P21796	26.68 32.96	30641	–0.419	0
Protein C14orf166	Q9Y224	26.68 27.50	28068	–0.462	0
Proteasome subunit alpha type 5	P28066	27.50	26394	–0.107	0
Peroxiredoxin 4	Q13162	27.50	30521	–0.218	2
Keratin, type II cytoskeletal 8	P05787	54.63	53510	–0,602	2
Glucose-6-phosphate 1-dehydrogenase	P11413	54.63	59097	–0,374	0
ERGIC-53 protein precursor	P49257	54.63	57713	–0.542	1
Endoplasmic reticulum protein ERp29 precursor	P30040	27.50	28975	–0.297	1
Triosephosphate isomerase	P60174	27.50	26522	–0.126	0
NADH-ubiquinone oxidoreductase 30 kDa subunit, mito precursor	O75489	27.50	30223	–0.292	1
Ras-related protein Rab-3D	O95716	27.50	24267	–0.345	0
Hypothetical prot CGI-109 precursor	Q9Y3B3	27.50	24354	–0.15	2
Guanine nucleotide-binding protein beta subunit-like protein 12.3	P25388	32.96	35055	–0.251	0
Electron transfer flavoprotein alpha-subunit, mitochondrial precursor	P13804	32.96	35058	0.146	1
Clathrin light chain A (Lca)	P09496	32.96	27060	–0.734	0
Elongation factor 1-delta	P29692	32.96 35.36	31103	–0.585	1

Table I. *continued.*

Protein name	SWISS-PROT accession number	Mr gel (kDa)	Protein MW (Da)	GRAVY value	No. of TMRs (TMPred)
Alpha-soluble NSF attachment protein	P54920	32.96	33246	–0.347	0
Malate dehydrogenase, mitochondrial precursor	P40926	32.96 35.36	35509	0.151	3
Voltage-dependent anion-selective channel protein 3	Q9Y277	32.96	30658	–0.28	1
Hypothetical protein KIAA0152	Q14165	32.96	32234	–0.156	3
Sideroflexin 1	Q9H9B4	32.96	35619	0.037	5
Voltage-dependent anion-selective channel protein 2	P45880	32.96	38092	–0.385	0
Guanine nucleotide-binding protein	P04901	35.36	37353	–0.231	1
Vesicular integral-membrane protein VIP 36	Q12907	35.36	40545	–0.364	2
Fructose-bisphosphate aldolase A	P04075	40.05	39264	–0.268	0
40S ribosomal protein SA	P08865	40.05 42.67	32854	–0.309	0
Actin, cytoplasmic 1 (Beta-actin)	P60709	40.05 42.67	41710	–0.2	2
Keratin, type I cytoskeletal 19	P08727	42.67	44079	–0.532	2
Cathepsin D	P07339	42.67	44552	0.023	2
Elongation factor 1-alpha 1	P04720	46.92	50109	–0.257	1
Keratin, type I cytoskeletal 18	P05783	46.92	47897	–0.561	2
Human elongation factor 1-gamma	P26641	46.92	50087	–0.481	2
Flotillin-1	O75955	46.92	47326	–0.338	1
Adipocyte plasma membrane-associated	Q9HDC9	46.92	46451	–0.186	1
Actin-like protein 3	P32391	46.92	47341	–0.266	2
eIF3 epsilon	O00303	46.92	37564	0.043	1
Protein disulfide isomerase A3	P30101	54.63	56747	–0.506	2
ATP synthase beta chain, mitochondrial	P06576	54.63	56625	–0.02	1
Tubulin alpha-1 chain (Alpha-tubulin 1)	P05209	54.63	50120	–0.230	1
Heat shock cognate 71 kDa protein	P11142	70.39	70854	–0.402	1
Stress-70 protein, mitochondrial	P38646	70.39	73635	–0.456	1

Digests from SDS-PAGE were analysed by nanoLC-MS/MS. Columns correspond to: protein name in Swiss-Prot; accession No. in Swiss-Prot; molecular weight measured on SDS-PAGE; molecular weight calculated from the sequence; grand average of hydrophobicity (GRAVY); number of transmembrane regions expected from the sequence according to the TMPred software.

and stained with silver nitrate. For protein identification, 200 µg of membrane proteins were loaded on 16-cm-long gradient gels (8-18% acrylamide), and stained with colloidal Coomassie blue (22). Both gels and running buffer contained 0.1% SDS. Electrophoresis (200 V constant voltage) were carried out using Laemmli SDS running buffer (0.025 M Tris-HCl, 0.192 M glycine, 0.005 M sodium thiosulfate, 0.1% w/v SDS) (23).

For 2-DE, the dried protein fractions were re-suspended in the rehydratation medium (16), with the addition of 2% Pharmalytes 5-8. Seventeen cm IPG strips were loaded either with 100 µg protein by cup-loading for analytical gels, or with 500 µg protein by in-gel passive rehydration for micropreparative gels in a PROTEAN IEF cell from Bio-Rad. Focalisation was achieved until 178,000 Vh was reached for analytical gels and until 195,000 Vh for preparative gels, respectively. The second-dimensional gels were run in a PROTEAN Plus Dodeca cell from Bio-Rad.

Protein visualization and gel analysis. Analytical gels were fixed for 60 min in 30% v/v ethanol and 5% v/v acetic acid and washed in water (10 min x 3). The gels were sensitized (0.02% w/v $Na_2S_2O_3$) for 1 min, washed for 2 min in water and incubated 30 min with silver solution (0.2% w/v $AgNO_3$, 0.01 v/v formaldehyde). Then, the gels were washed for 5-10 sec with water and developed in 2.4% w/v Na_2CO_3, 0.01 v/v formaldehyde, 0.0013% w/v $Na_2S_2O_3$. The development was stopped in 0.33 M Tris-acetic acid, pH 7.4. Micropreparative 1-D and 2-DE were stained with Colloidal Coomassie blue. The stained gels were digitized using a GS-700 densitometer from Bio-Rad and the images were analysed with Image Master 2D Platinum software (GE Amersham Biosciences) (5, 20, 21, 24).

Proteolysis and mass spectrometry. In-gel trypsin digestion was carried out using the Amersham Ettan Digester. The peptides extracted from 1-D gel were analysed by Agilent 1100 series nanoLC-MS/MS (Ion trap) equipped with a nanospray ion source (Agilent Technologies, Santa Clara, CA, USA). Prior to ion trap analysis, the samples were desalted and concentrated using a C18 Zorbax 300SB enrichment column (5 mm x 300 µm, 5 µm). A 60-min gradient (flow rate, 0.3 µl/min) from 2-80% B was used where solvent A was 0.1% v/v aqueous formic acid in 2%v/v acetonitrile and solvent B was 0.1% v/v aqueous formic acid in 98% v/v acetonitrile. Using the Mascot Server software package with Mascot Daemon client application (Matrix Science Ltd., London,

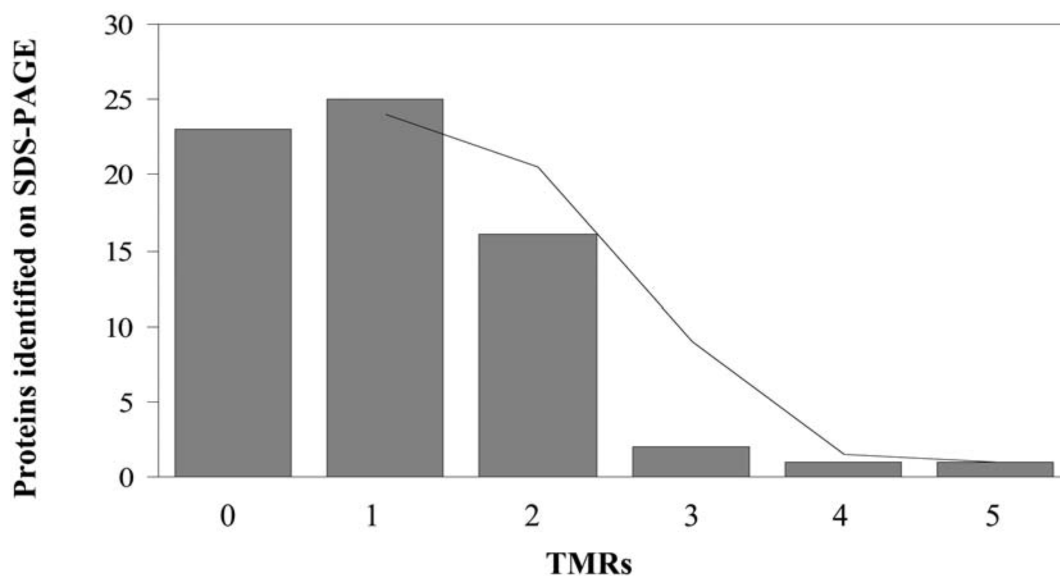

Figure 2. *Proteins identified in the membrane fraction resolved by SDS-PAGE. Distribution of TMRs according to the TMPred algorithm.*

UK), tandem mass spectra of tryptic digest peptides were searched against SwissProt/TrEMBL databases, which are accessible at http://www.expasy.org/sprot/. Prediction of transmembrane regions (TMRs) was conducted using the TMPred program (URL: http://www.ch.embnet.org/software/TMPRED_form.html).

Results

Protein pattern analysis of the membrane extract. A flow chart of the methods used in this work is depicted in Figure 1. The selectivity of the procedure was first investigated by 1-DE protein profiling. Sequential gel plugs corresponding to proteins with molecular weight from 16109 to 70369 kDa were subjected to trypsinolysis, and the resulting peptide fragments analysed by tandem mass spectroscopy (MS/MS). Sixty-eight distinct proteins were identified by Mascot from 13 gel plugs (Table I). The migration distance and the theoretical molecular weight of the identified MCF7 proteins were found to correlate quite well for most proteins. The subcellular location of the identified proteins indicate that the membrane preparation procedure employed in this study also contained membranes from endoplasmic reticulum, golgi apparatus and mitochondria. As a first insight, the calculation of grand average of hydrophobicity (GRAVY) scores (25) showed that 16% of the proteins displayed positive scores, with values up to 0.168. It confirms that hydropathy calculation alone does not result in a reliable prediction of membrane proteins (5, 26). Further, the occurrence of TMRs was predicted using the TMPred algorithm. Approximately 30% of the identified proteins were expected to possess at least 2 TMRs, including a protein with 5 TMRs (sideroflexin 1), and this proportion dropped to 66% for proteins with at least 1 TMR (Figure 2).

Two-DE analysis. To evaluate if the cancer cell membrane protein preparation used in this study was suitable for 2-DE, the spot patterns of three sets of gels corresponding to whole cell, membrane and soluble extracts were compared. Cross-matching and statistical analysis were performed for each set of gels and between the different sets to highlight both the reproducibility of the extraction process and its usefulness for 2-DE analysis. Figure 3 shows representative gels of the three fractions. All three fractions gave rise to good quality 2-DE, and each fraction led to a distinct spot pattern. To evaluate the reproducibility of the TFE-extraction method, two membrane fractions and two soluble fractions were prepared in independent experiments (Figure 4). Scatter plots confirmed the gels similarity for the two extractions (correlation coefficient >0.917). This goodness-of-fit allowed the conclusion that the extraction and separation conditions of the membrane and soluble fractions were reproducible. Close patterns were detected for each set of gels (635-845 spots, and 1427-1477 spots, for the membrane fractions and the soluble fractions, respectively). The cross-matching between membrane and soluble protein gels, and between them and the gels of the whole-cell extract, are presented in Table II. For each condition of extraction, high levels of matching were found (Soluble 1/Soluble 2; Membrane 1/Membrane 2). On the other hand, the analysis impressively highlighted the differences between the three fractions. As expected, 83% of the spots on the gels corresponding to the membrane fraction were not found on the gels of the soluble

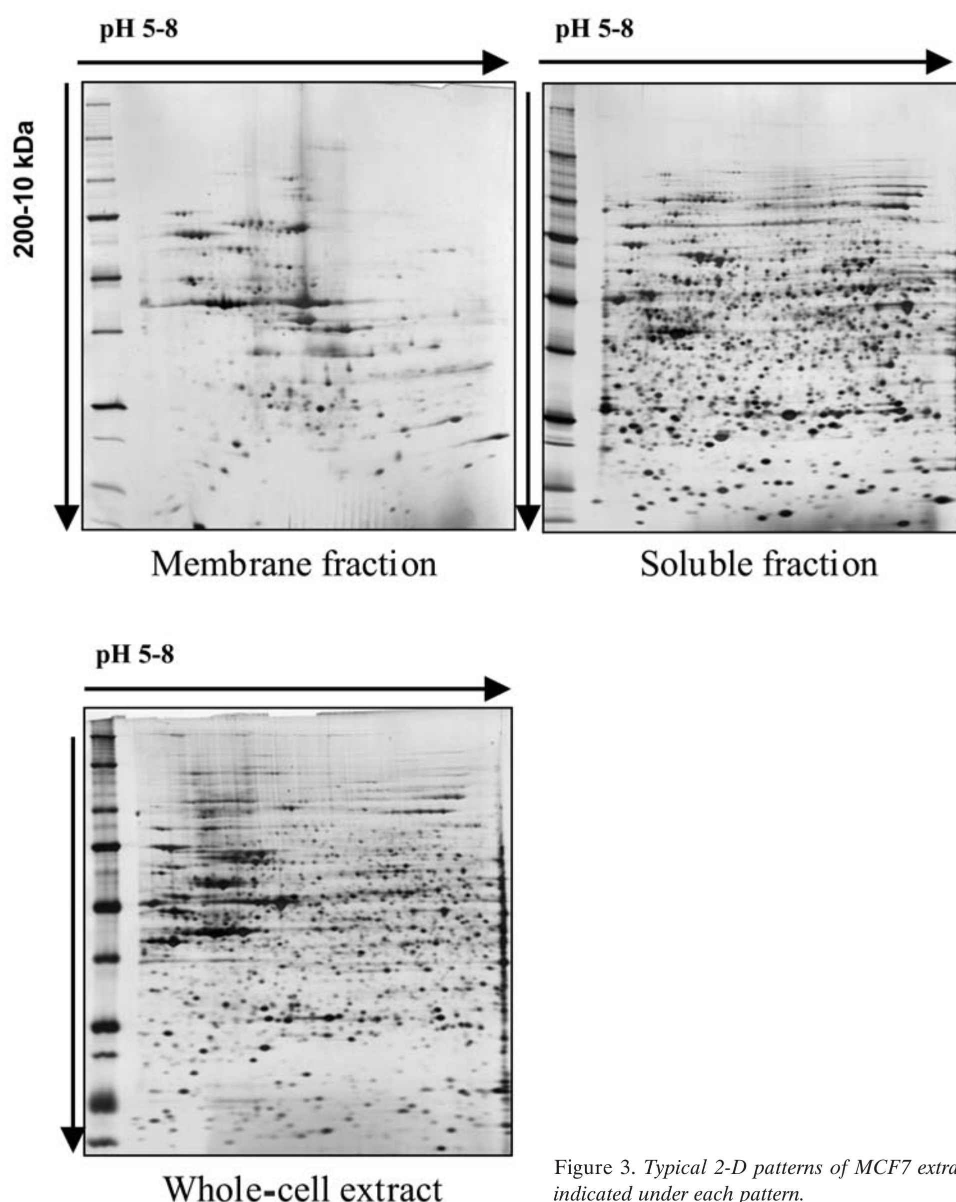

Figure 3. *Typical 2-D patterns of MCF7 extracts. The type of extract is indicated under each pattern.*

Table II. *Comparison of image analysis data of whole-cell, soluble and membrane extracts.*

Gel 1	Gel 2	Number of matches	Percent matches
Soluble 1	Soluble 2	1201	80.63
Membrane 1	Membrane 2	484	65.49
Soluble	Membrane	162	16.62
Soluble	Whole-cell	903	66.05
Membrane	Whole-cell	98	11.47

Soluble 1 and 2, and Membrane 1 and 2 correspond to two independent extractions. For matching between the different sets of gels, the gel with the higher number of spots was chosen for each set.

fraction (Soluble/Membrane). Spots detected either in the soluble or in the membrane fractions were detected in part in the whole-cell protein gel. This was particularly true for the soluble proteins. Indeed, the percentage of matching between whole-cell protein gels and soluble fraction gels was around 66%. In the whole-cell extract, competition between high-solubility and low-solubility proteins probably prevented both the extraction and the 2-D resolution of more hydrophobic proteins. This can, for example, be observed in the boxed areas of the gels shown in Figure 5, where boxes highlight proteins extracted only in the membrane fraction.

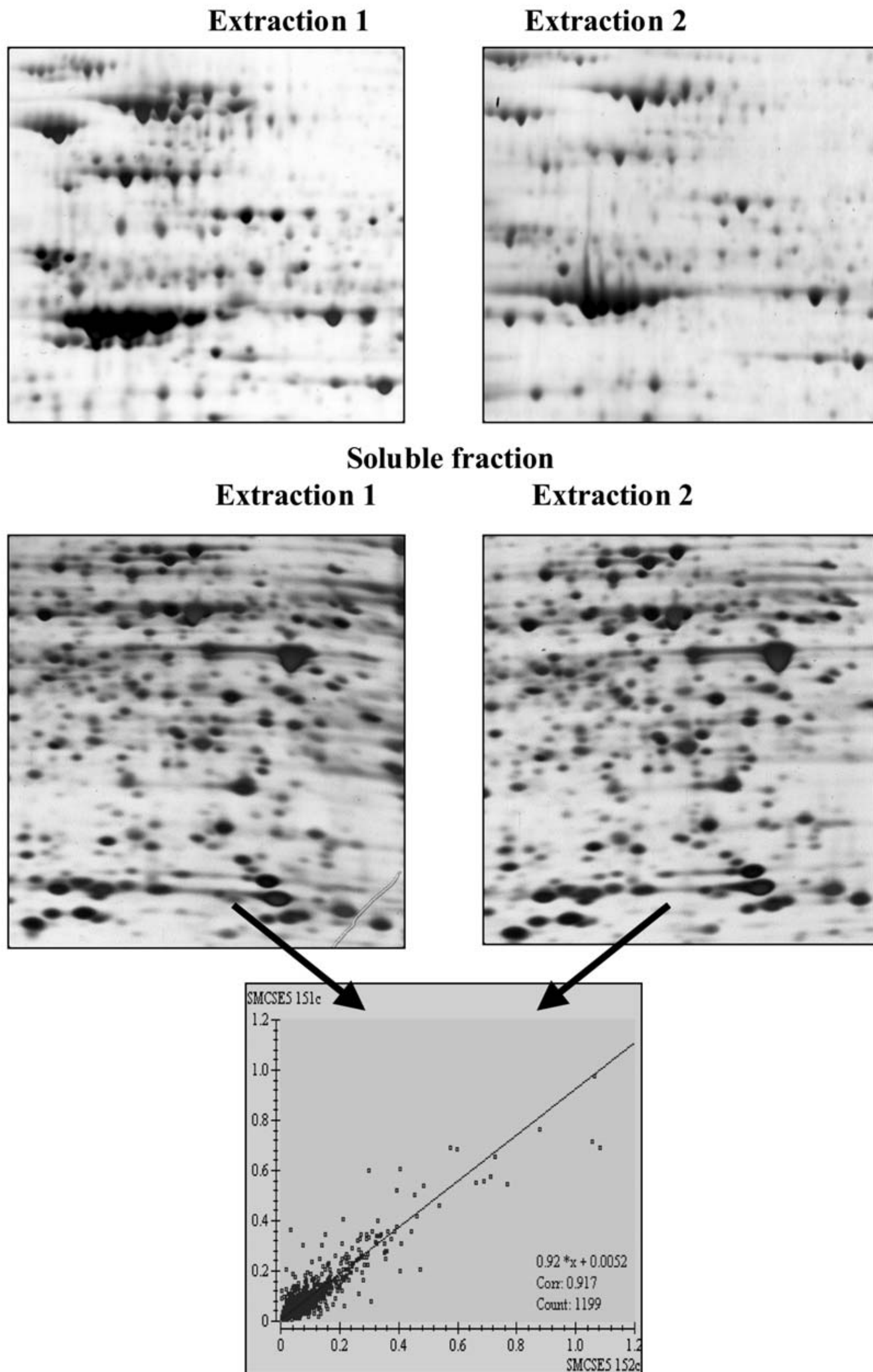

Figure 4. *Comparison of 2-D patterns on membrane and soluble fractions, for two independent extractions. The analysis of a representative window shows the similarity between the patterns in each pair of gels (>75%). This similarity was confirmed by scatter plots showing the correlation coefficient and the regression line equation (shown for the soluble fraction; Corr: 0.917).*

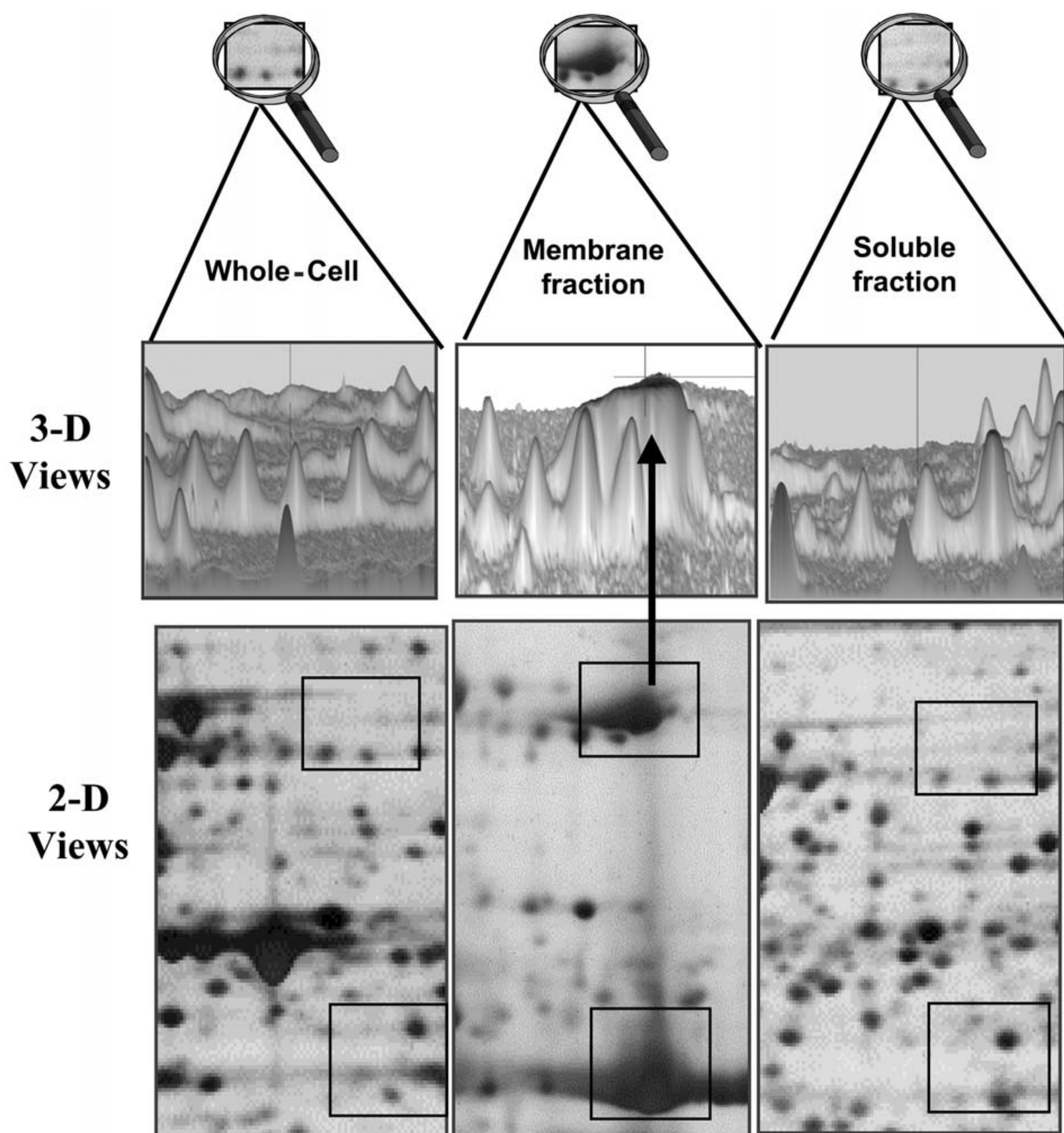

Figure 5. *Examples of 2-DE separations allowing the detection of proteins specific to the membrane extract. 2-D and 3-D views of the 2-DE gels confirm the absence of most membrane proteins in the whole-cell and soluble extracts, respectively.*

Discussion

The usefulness of a proteomic approach to identify relevant membrane or membrane-associated proteins has been established (4). Nevertheless, prerequisites for the success of membrane proteomics are standardized and reproducible operating procedures for sample preparation. The purpose of this study was to evaluate a procedure for human cancer cells suitable for 2-DE. It was important first to minimize the number of sample preparation and separation steps in order to minimize sample loss and, second, to validate these steps on a cell model. Due to its simplicity and efficiency, the extraction and separation approach described here appears well suited for membrane proteomic investigations of human cancer cells, allowing the 2-D separation of potential biomarkers undetected by current techniques.

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