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Comparative proteomics of rat liver and Morris hepatoma 7777 plasma membranes☆

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Abstract

Plasma membranes from normal rat liver and hepatocellular carcinoma Morris hepatoma 7777 were selectively solubilized by use of different reagents. After selective solubilization, proteins were identified by nano-HPLC–electrospray ionization tandem mass spectrometry (LC-ESI MS/MS). Using simple software, the patterns of proteins identified in membrane solubilizates from liver and hepatoma were compared. Proteins identified in Morris hepatoma 7777 and not in the corresponding membrane solubilizate from liver, mostly members of the annexin and heat shock protein families, are discussed as potential candidate markers for hepatocellular carcinomas.

Keywords: Liver; Morris hepatoma 7777; Plasma membrane; Mass spectrometry; Candidate biomarker

1. Introduction

Plasma membranes play crucial roles in cell function. Membrane proteins and other components, mainly glyco- and phospholipids, are involved in receptor-binding and further transport of bound components into the cell. They are also involved in cell–cell and cell–matrix interactions, in the organization of the cytoskeleton, and they determine immunological identity of the cell [1–3]. The composition and antigenicity of membrane proteins of tumor cells are altered during malignant transformation [3]. Antigens on the cell surface are the first ones that will provoke a reaction by the host immune system. Additionally, the possibility that these proteins, or fragments thereof, are the first ones to enter the blood stream, e.g. through abrasion, makes them likely candidates for cancer biomarkers [4,5].

Depending on the type of interaction, membrane proteins are either embedded in the lipid bilayer or associated with peripheral membrane structures; in the later case, usually by ionic interactions or by hydrogen bonds. In the early eighties, methods for selective solubilization of membrane proteins by use of salts, chaotropic reagents, and different detergents were developed [6–9]. We have adapted these methods for the solubilization of membrane proteins from normal liver and a group of chemically-induced liver carcinomas, Morris hepatomas [10]. In the first step, loosely associated proteins are solubilized by repeated freezing and thawing of plasma membranes. Membrane-associated and peripheral proteins are removed by use of different salt solutions or high pH (pH 11) reagents. In the third step, more hydrophobic, integral membrane proteins are solubilized by use of different detergents (6–10). Lastly, some detergent-insoluble proteins are extracted by calcium chelation with EDTA or EGTA in the presence of a detergent, such as octyl glucoside or CHAPS [8,10].

Recently, we analyzed proteins solubilized with EGTA from the detergent-resistent pellet from normal liver and hepatocellular carcinoma Morris hepatoma 7777 by use of proteomic methods. Striking differences between liver and this highly malignant carcinoma in their protein patterns were found [11]. The main component extracted by EGTA from normal liver plasma membranes is the calcium binding protein annexin A6. In Morris hepatoma 7777 plasma membranes, this protein is accompanied by the low molecular weight members of the annexin family. The possible use of low molecular weight annex-

Abbreviations: F/T, freeze/thaw; Tx100, Triton X100; NL, normal liver; M Hep, Morris hepatoma 7777

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ins as biomarkers for hepatocellular carcinomas was discussed [11].

In the present paper, we have analyzed additional fractions from the solubilization of plasma membranes from rat liver and Morris hepatoma 7777. Their patterns were compared to find differences in protein expression between the normal tissue and this highly malignant hepatocellular carcinoma.

2. Material and methods

2.1. Isolation of plasma membranes

Rat liver and Morris hepatoma 7777 plasma membranes were isolated by zonal centrifugation using a Kontron centrifuge (Kontron Analytik, Munich, Germany) as described elsewhere [12]. Membrane purity was routinely checked by electron microscopy and marker enzyme assays [13].

2.2. Selective solubilization of plasma membranes

Plasma membranes were solubilized according to the method of Josic and Zeilinger [10]. The membranes were first frozen at −80 °C and thawed. After centrifugation, the supernatant (F/T-solubilizate) was separated, and the pellet extracted with 20 mM Na_2CO_3, pH 11. After homogenization (Dounce homogenizer at 25 °C) and centrifugation at 100,000 × *g*, the supernatant (pH11-solubilizate) was decanted and the pellet extracted with 1% Triton X100 (Sigma, St. Louis, MO, USA) in Tris buffered saline, pH 7.2. After homogenization (Dounce homogenizer at 4 °C) and centrifugation at 100,000 × *g*, the supernatant (TX100-solubilizate) was decanted. In the last step, the remaining membrane pellet was extracted with 25 mM EGTA, pH 7.4, containing 1% (w/v) octyl glucopyranoside (Sigma) [8,11].

2.3. Protein determination

Protein content of the membrane preparations and different membrane extracts was determined using the BCA protein assay kit from Pierce, following the manufacturer's procedure (Pierce, Rockfort, IL, USA). Bovine serum albumin was used as standard.

2.4. Trichloroacetic acid precipitation

Before tryptic digestion of detergent containing fractions, protein samples were cleaned up by precipitation with trichloroacetic acid (TCA). TCA precipitation was performed using Bio-Rad's Ready Prep™ 2D cleanup kit (BioRad Laboratories, Richmond, CA, USA).

2.5. Tryptic digestion

Ten micrograms aliquots from the various protein extracts were digested with TPCK-treated bovine trypsin (Sigma). If a sample contained Triton X-100, the protein was first precipitated with the ReadyPrep™ 2D Cleanup Kit, following manufacturer's instructions (Bio-Rad Laboratories, cf. above). Prior to digestion, samples were diluted to 40 μL with 100 mM ammonium bicarbonate, pH 8.1, heat-denatured for 10 min at 90 °C, and then rapidly cooled on ice. The extracts were digested with 100 ng trypsin for 4–6 h at 37 °C. A second 100 ng aliquot was then added, and the incubation continued overnight. The digestions were terminated by addition of 5 μL of 5% (v/v) formic acid/50% (v/v) acetonitrile. After 10 min at room temperature, the digests were centrifuged to pellet any insoluble material.

2.6. Protein identification by LC–MS/MS

LC–MS of tryptic digests was performed as described previously [14], with slight modifications. Most importantly, because injections using the equivalent of 1 μg of starting protein were performed, a very shallow (5–35% solvent B over 120 min) acetonitrile gradient was used, and MS data collection was extended to 4 h. Eluting peptides directly entered the QSTAR XL hybrid qTOF mass spectrometer (Applied Biosystems, Foster City, CA and Sciex, Concord, Ontario, Canada) via electrospray ionization.

The parameters used to collect MS and MS/MS spectra, using standard information dependent acquisition (IDA) methods, were as described previously [11]. All protein identifications were performed with ProteinPilot software (Applied Biosystems and Sciex). Experimental spectra were matched against *in silico* tryptic digests of the entire NCBI nr database (7 July 2006), using the "generic workup" and "biological" modification sets provided with this software package. The "generic workup" set contains 35 modifications that might occur during the handling of protein/peptide samples, such as oxidation, dehydration and deamidation. The "biological" set consists of 94 possible modifications, including acetylation, methylation and phosphorylation. ProteinPilot provides a percent confidence for the agreement between the experimental and theoretical fragmentation patterns. However, it limits the confidence of its assignments to 99%. Confidences are converted to peptide scores using the formula, $-\log_{10}((100 - \%\ \text{confidence})/100)$; an assignment at 99% yields a score of two. A protein's score (*S*) is the sum of confidence values for independent, "sequence distinct" peptide assignments. Because the score is derived from completely independent observations, it is a measure of the likelihood that the protein assignment is incorrect; specifically, a protein assignment may be wrong once every 10^S times. ProteinPilot automatically clusters the identified proteins into groups that share common peptides. The results were parsed with in-house software to keep only rat proteins, as annotated in the nr database. Specifically, retained proteins were restricted to those having "rat" or "Rattus" in either the species or the protein name.

LC–MS/MS protein identifications were done from at least two samples from independent preparations of plasma membranes from normal liver and Morris hepatoma 7777.

3. Results

3.1. Extraction of plasma membranes and separation and identification of proteins

As an example of experimental replicates, Fig. 1 shows the comparison between two analytical runs of two independent

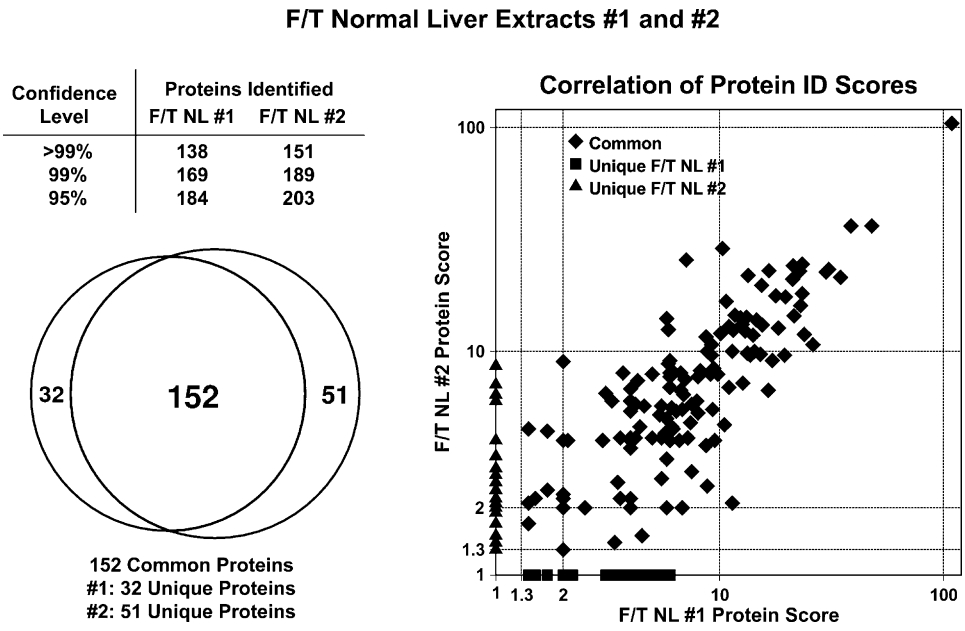

Fig. 1. Numbers of proteins identified in two different runs from two different normal liver (NL) plasma membrane preparations. Analyzed proteins were solubilized by freezing and thawing (F/T). The total number of ProteinPilot identifications, using the entire NCBI nr database, but after filtering out all non-rat proteins (as annoted in nr), at three different confidence levels is shown in the table in the upper left. The Venn diagram illustrates the overlap between 95% confidence proteins. The log–log plot of protein scores reveals the correlation between experimental samples. ProteinPilot scores (S) are related to confidence (C) by the formula: $C = 100 \times (1 - 10^{-S})\%$. Therefore, the lines at $S = 1.3$ and 2.0 demark the 95% and 99% confidence thresholds. Because no peptide assignment can contribute more than two to a protein score, those protein identifications above 2.0 (>99% in the table) must have two or more peptides contributing to the identification. *Note*: To place the unique proteins on the log-scaled graph, their protein scores were arbitrarily set to 1.

liver plasma membrane preparations (F/T-solubilizate). Typically, between 150 and 250 proteins are identified with a score higher than 1.3 (identification with more than 95% probability) in each LC–MS run. As shown in Fig. 1, 152 of the identified proteins are present in both samples. 32 proteins out of 184 (17%) were unique to replicate 1 and 51 out of 203 (25%) proteins were unique to preparation 2. The variation in the analyzed specimens of proteins identified, independent of the kind of solubilizate (F/T-, pH11- and TX100-solubilizate), was between 15 and 25% (data not shown).

Fig. 2 shows a typical total ion chromatogram for a nano-HPLC separation of a tryptic digest of the TX-100-solubilizate from Morris hepatoma 7777. A representative MS/MS fragmentation spectrum and the identification of the peptide NLLHVTDTGVGMTR, belonging to tumor rejection antigen gp96, is shown in Fig. 3. All peptides that belong to gp96 and

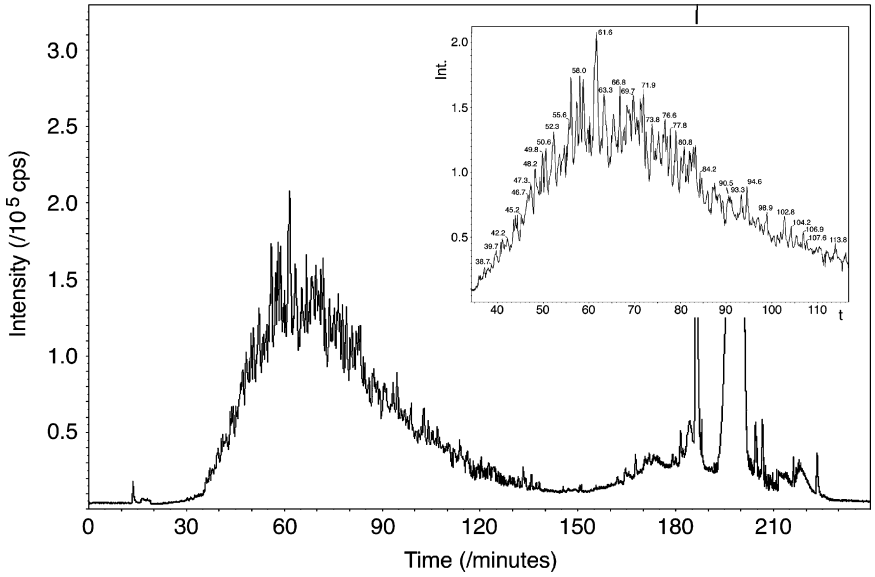

Fig. 2. Protein identification by nano-HPLC-ESI MS/MS: representative total ion chromatogram of the tryptic digests of the various membrane extracts. Over 10,000 scans were collected by the mass spectrometer during this 4 h HPLC run of the digest of TX100-soluble proteins from Morris hepatoma 7777. Inset, magnified view of the chromatogram from 35 to 115 min, when most of the tryptic peptides elute.

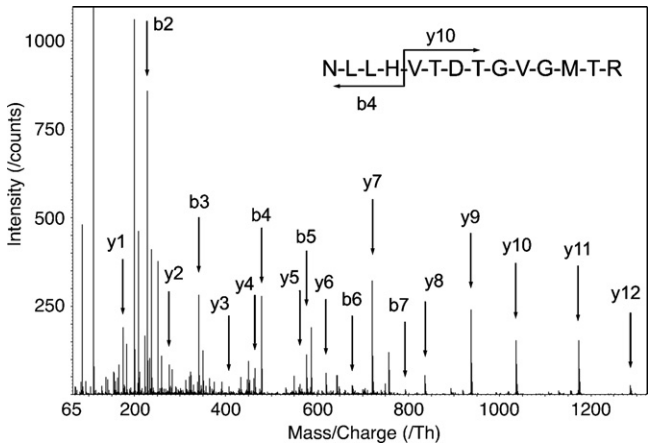

Fig. 3. One of the fragmentation spectra collected during the LC–MS/MS run shown in Fig. 2. This fragmentation pattern was assigned with the maximally allowed confidence (99%) to the sequence NLLHVTDTGVGMTR from tumor rejection antigen gp96. The cleavage location that generates the b_4 and y_{10} ions is displayed along the sequence. Some of the ions' assignments to *b*- and *y*-series fragments are labelled.

that were identified by LC–MS/MS in this run are listed in Table 1.

3.2. Solubilizate after freezing and thawing

The comparison between identified proteins in the solubilizates after freezing and thawing of normal liver and Morris hepatoma 7777 plasma membranes is summarized in Fig. 4.[1] In two parallel runs, 184 proteins were identified in the F/T-solubilizate from the liver plasma membranes, 60 of which are in common with the proteins identified in the F/T-solubilizate from Morris hepatoma 7777. In the Morris hepatoma 7777 solubilizate, of 167 proteins that were identified, 107 are unique to these samples of this liver carcinoma.

Table 1
MS/MS identification of rat tumor rejection antigen gp96 in the Triton X100 solubilizate from Morris hepatoma 7777 plasma membranes

Peptide sequence	% Confidence	Contribution	ΔMass (/Da)
NLLHVTDTGVGMTR	99	2.00	−0.003
EEEAIQLDGLNASQIR	99	2.00	−0.019
GVVDSDDLPLNVSR	99	2.00	−0.012
RVFITDDFHDMMPK	99	2.00	0.004
EEASDYLELDTIK	99	2.00	0.006
VFITDDFHDMMPK	99	2.00	0.004
IADEKYNDTFWK	99	2.00	0.010
FQSSHHSTDITSLDQYVER	99	2.00	−0.001
TVWDWELMNDIKPIWQRPSK	99	2.00	−0.007
ELISNASDALDK[a]	94	1.22	0.993
KYSQFINFPIYVWSSK	89	0.96	−0.020
FAFQAEVNR	86	0.85	0.004
SILFVPTSAPR	75	0.60	−0.004
YSQFINFPIYVWSSK	70	0.52	−0.013
YNDTFWK	16	0.08	0.003
LIINSLYK	15	0.07	0.001
EFEPLLNWMK	10	0.05	−0.006
EATEKEFEPLLNWMK	2	0.01	0.033

Ions assigned to gp96 tryptic peptides, based on the agreement between the expected and experimental fragmentation results, are listed by decreasing confidence in the assignments. The contributions ($-\log_{10}[(100 - \%\ \text{confidence})/100]$) of the peptide assignments to the overall protein score for gp96 are listed in the second column. The mass error for the parental ion is shown in the "ΔMass" column. The experimental fragmentation spectrum for the first peptide (NLLHVTDTGVGMTR) is shown in Fig. 3.

[a] The experimental MS/MS evidence supports deamidation ($\Delta m_{\text{theor}} = 0.984$ Da) of asparagine #5.

3.3. Solubilizate after extraction at pH11

Fig. 5[2] shows the comparision of the pH11 membrane solubilizate from liver and Morris hepatoma 7777. In the pH11-solubilizate from rat liver, 150 proteins were identified, with 75 being unique to this organ. In the solubilizate from the tumor plasma membranes, 191 proteins were identified. Seventy-five of these proteins are in common with the corresponding solubilizate from the liver plasma membrane preparation, and 116 proteins are unique to this tumor.

3.4. Solubilizate after extraction with Triton X100

The comparison between proteins identified in the TX100-solubilizate from liver and Morris hepatoma 7777 plasma membranes is shown in Fig. 6[3]. In these samples, 91 common proteins from liver and this tumor were identified. In the TX100-solubilizate from the liver plasma membranes, 166 proteins out of 257 identified proteins are unique to this organ. In the corresponding solubilizate from the Morris hepatoma 7777 plasma membranes, 160 proteins out of 251 identified proteins are unique for this tumor.

4. Discussion

Selective solubilization by use of different reagents, combined with nano-HPLC and ESI MS/MS, is a powerful tool for the analysis of integral and membrane associated proteins from rat liver and Morris hepatoma 7777. As shown here (cf. Fig. 1), the results are reproducible, and reliable detection of differences between isolated plasma membrane fractions from normal and malignant tissue is possible.

As shown in Fig. 4, out of 184 identified proteins, only 60 (33%) normal liver proteins are in common with proteins identified in the F/T solubilizate from Morris hepatoma 7777. Similarly, only 36% (60/167) of the identified proteins in the hepatoma F/T solubilizate are in common with the proteins identified in the F/T solubilizate from normal liver plasma membranes. 107 proteins (64%) are unique to the plasma membrane solubilizate from this hepatocellular carcinoma. These results are not surprising, given the altered metabolic activity and cell surface properties of this rapidly growing and highly metastatic

[1] Complete lists of identified proteins are available electronically as supplements (Tables 1S, 2S and 3S.).

[2] The complete list of identified proteins is available electronically as supplementary material (Tables 4S, 5S and 6S.).

[3] A complete listing of identified proteins is available electronically as a supplement (Table 7S, 8S and 9S.).

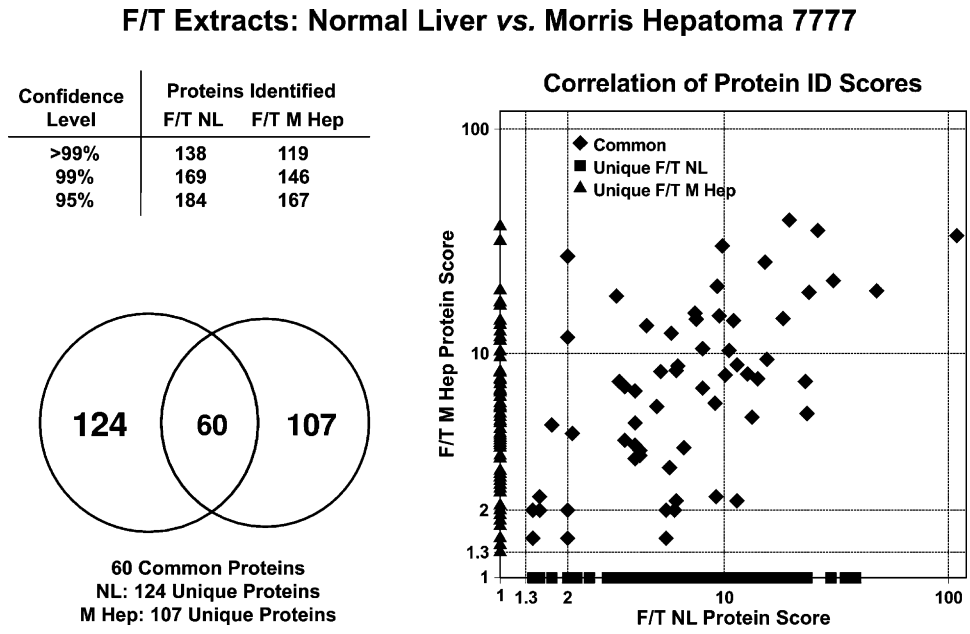

Fig. 4. Comparison of proteins identified in solubilizates after freezing and thawing (F/T) from normal liver (NL) and Morris hepatoma 7777 (M Hep) plasma membranes. See Fig. 1 for details.

carcinoma [15]. Some of the proteins found only in the F/T solubilizate from Morris hepatoma 7777 are listed in Table 2. Alpha glucosidase II [16] and ERO1 [17] are both involved in the processing of proteins transiting through the endoplasmic reticulum. Additionally, ERO1 has been shown to be upregulated in the hypoxic environment of human tumors [18]. α-Fetoprotein, cadherin, glutathione *S*-transferase π, and aldolase A are well-known cancer markers [19–24]. Additionally, α-fetoprotein is a "classical" biomarker for hepatocellular carcinomas [19]. Cadherin is an essential component of adherence junctions. The loss of these junctions leads to enhanced proliferation, motility and finally metastasis, all behavior typical for malignant cells [22]. Cadherin has already been discussed as a biomarker for several types of cancer [23,24]. Coronin, profilin and cofilin 1 are all involved in actin polymerization and de-polymerization [25–27]. Overexpression of these proteins may be a consequence of the altered cytoskeletal structure in Morris hepatomas [15]. CD44 is a multistructural and multifunctional cell surface molecule involved in cell proliferation, cell differentiation, cell migration, angiogenesis, et cetera. In many cancer types, high level CD44 expression is associated with unfavorable clinical outcome [28]. Heat shock proteins belong to a group of highly

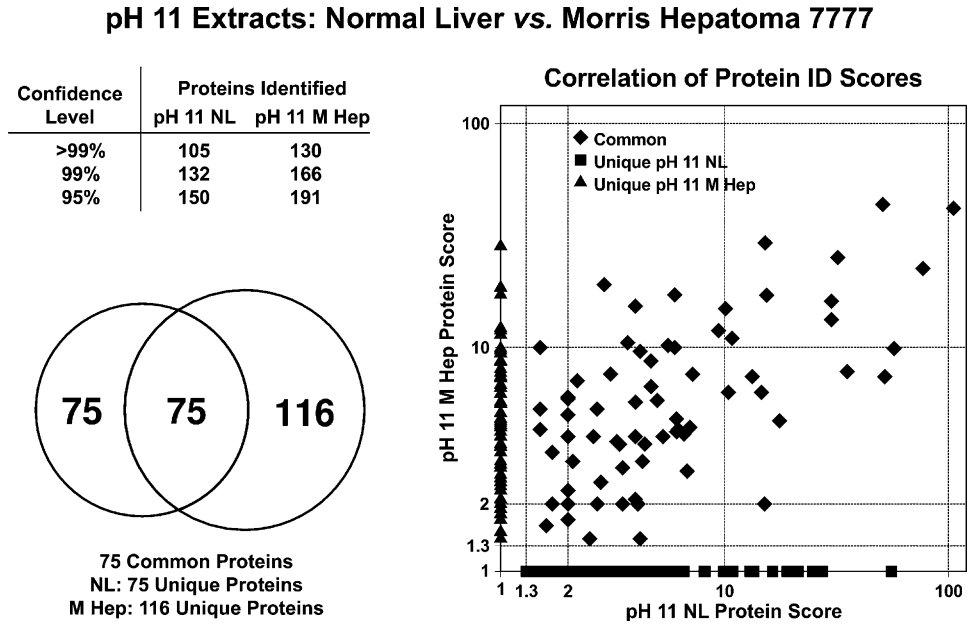

Fig. 5. Comparison of proteins identified in solubilizates from liver (NL) and Morris hepatoma 7777 (M Hep) after treatment at pH11. The details are as described in Fig. 1.

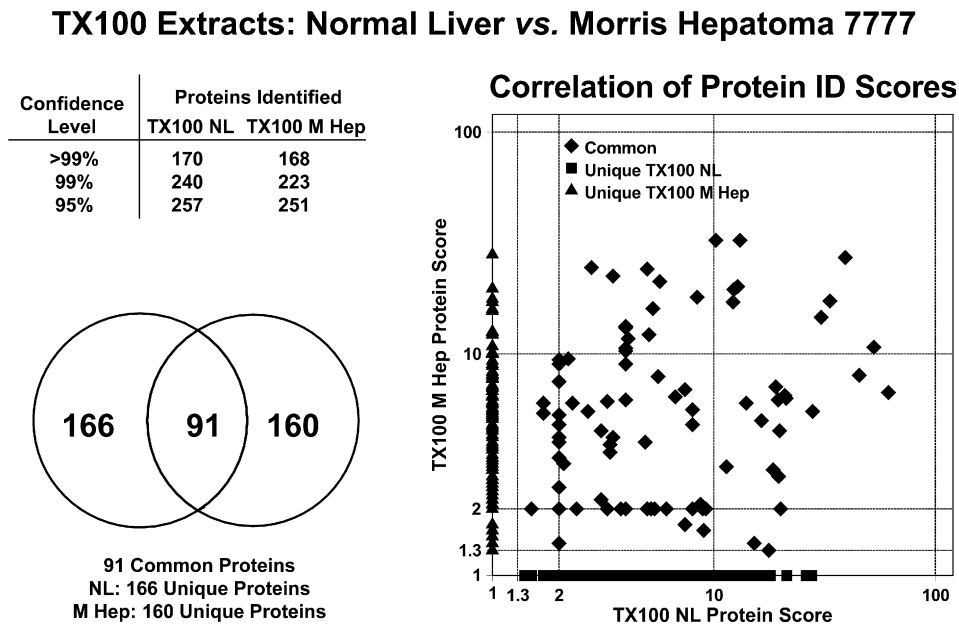

Fig. 6. Comparison of proteins identified in Triton X100 (TX100) solubilizates from liver (NL) and Morris hepatoma 7777 (M Hep) plasma membranes. See Fig. 1 for details.

conserved proteins with different cellular localizations [29]. Some of these proteins have been discussed recently as biomarkers for hepatocellular carcinomas [19]. Shin et al. [30] identified proteins with chaperone function in high concentrations on the surface of several cancer cells. This group of proteins includes heat shock proteins 70, 60, 54 and 27, glucose regulated proteins 78 and 75, and protein disulfide isomerase. Oncomodulin, a typically cytoplasmic, calcium binding protein is highly tumor-specific, and has been detected in many chemically induced rat hepatomas [31]. The presence of oncomodulin in the plasma membrane fraction may be the result of calcium-dependent association with other membrane bound proteins.

Figs. 5 and 6 present the numbers of proteins that are differentially identified in the pH11- and TX100-solubilizates, respectively, from normal liver and Morris hepatoma 7777. Out of 191 identified proteins, 116 proteins (61%) are unique to the pH11 Morris hepatoma 7777 plasma membrane extract. Proteins differentially identified in the pH11-solubilizate from Morris hepatoma 7777 that are not identified in the corresponding solubilizate from normal liver are listed in Table 3. In the TX100-solubilizate, out of 251 identified proteins, 160 (64%) were unique to Morris hepatoma 7777 (cf. Fig. 6). Table 4 lists a selection of proteins found only in the Morris hepatoma 7777 extract.

As shown in Tables 3 and 4, an additional 10 heat shock proteins could be identified in two of the plasma membrane fractions from Morris hepatoma 7777, specifically the pH11- and TX100-solubilizates. We had already identified six of these

Table 2
Selected proteins identified in the F/T extract from Morris hepatoma 7777 plasma membranes that are absent in the extract of normal liver plasma membranes

Protein name	Score[a]	Cover[b]	Peptides[c]	Accession[d]
α-Fetoprotein	36.8	35.0	30	gi|6978471
Similar to α-glucosidase II, α subunit	31.7	28.8	25	gi|62641851
Cadherin	19.1	25.8	15	gi|505563
Glutathione *S*-transferase, π2	16.9	53.3	10	gi|34849843
Heat shock protein 1α	14.0	24.0	13	gi|54673763
Aldolase A	11.8	34.6	9	gi|6978487
Endoplasmic oxidoreductase 1 (ERO1-like)	10.2	26.3	9	gi|19744821
Profilin 1	6.8	37.1	4	gi|42476144
Coronin, actin binding protein 1A	6.0	9.8	3	gi|18426834
Heat shock 90 kDa protein 1β	4.4	22.1	4	gi|51859516
Cofilin 1	3.5	24.1	3	gi|8393101
CD44 protein	2.0	3.3	1	gi|38181806
Oncomodulin	1.7	11.0	1	gi|39930606

[a] The score (*S*) is a measure of the confidence in the assignment. Specifically, the relative certainty is $100 \times (1 - 10^{-S})$ percent: a score of 1.3 implies 95% confidence, 2.0 indicates 99%, 4.0 signifies 99.99% confidence, etc.

[b] Cover (sequence coverage) is the percentage of the protein sequence that was identified by MS/MS peptide assignments.

[c] Peptides refers to the number of sequence-distinct peptides whose assignment contributed to the protein's score.

[d] NCBI Entrez protein accession number.

Table 3
Selected proteins identified in the pH11 extract from Morris hepatoma 7777 plasma membranes that are absent in the pH11 extract of normal liver plasma membranes

Protein name	Score[a]	Cover[b]	Peptides[c]	Accession[d]
Heat shock 70 kD protein 5	28.3	33.6	18	gi|38303969
Annexin A4	18.5	43.9	12	gi|55742832
Tumor rejection antigen gp96	12.2	21.5	12	gi|58865966
CaBP1	11.9	30.2	8	gi|488838
Glucose regulated protein, 58 kDa	11.4	21.2	8	gi|38382858
Annexin 5	9.9	37.9	11	gi|51858950
Annexin 1	8.0	19.7	4	gi|6978501
Transferrin receptor	6.6	17.1	8	gi|62658000
Heat shock 90 kDa protein 1β	6.2	15.1	8	gi|51859516
α-Fetoprotein	4.8	11.1	4	gi|809077
Heat shock 10 kDa protein 1	3.3	43.1	4	gi|6981052
S100 calcium binding protein A10	4.2	35.8	3	gi|13592079
Heat shock protein 1	4.0	4.2	2	gi|11560024
Basigin 2	2.6	7.2	2	gi|33350936
Annexin A7	2.1	7.8	2	gi|18426844
Annexin A11	2.4	4.0	2	gi|53734394
Glutathione *S*-transferase, π 2	2.0	16.7	1	gi|34849843
Calnexin	1.5	2.5	1	gi|310085

[a] The score (*S*) is a measure of the confidence in the assignment. Specifically, the relative certainty in the identification is $100 \times (1 - 10^{-S})$ percent.
[b] Percent sequence coverage.
[c] Number of sequence-distinct peptides whose assignment contributed to protein's score.
[d] NCBI Entrez protein accession number.

proteins in highly purified plasma membranes from another hepatocellular carcinoma cell line (heat shock 90 kDa protein 1β; heat shock protein 1; heat shock protein 1α; glucose regulated protein 58 and calnexin, cf. [32]). The overexpression of members of the heat shock protein 70 families, glucose regulated proteins 78 kDa and 75 kDa, HSC70 and heat shock 70 kDa protein 1 has also been found by comparative 2D-electrophoretic analysis of liver tissue and hepatocellular carcinomas associated with Hepatitis C in Japanese patients [33]. Overexpression of these proteins, together with heat shock protein 27 (Hsp 27), was also found by comparative 2D-electrophoretic analyses of liver and hepatocellular carcinomas from Taiwanese [34] and

Table 4
Selected proteins identified in the TX100-extract from Morris hepatoma 7777 plasma membranes that are absent in the TX100-extract of normal liver plasma membranes

Protein name	Score[a]	Cover[b]	Peptides[c]	Accession[d]
Transferrin receptor	19.7	27.9	17	gi|62658000
Cadherin	17.7	23.3	12	gi|505563
Aldolase A	8.8	23.4	7	gi|6978487
Hsc70-ps1	8.3	19.5	7	gi|56385
Annexin 5 (Lipocortin V)	8.0	21.6	5	gi|51858950
Annexin A2	7.7	22.7	5	gi|9845234
Annexin A4	6.5	20.4	3	gi|55742832
UDP-glucose ceramide glucosyltransferase-like 1	6.0	4.6	3	gi|19424302
α-Fetoprotein	6.0	13.4	6	gi|6978471
Peroxiredoxin 3	5.2	14.4	3	gi|11968132
Glutathione *S*-transferase (μ1)	4.3	17.0	3	gi|8393502
Heat shock 10 kDa protein 1	4.2	33.3	4	gi|6981052
Transmembrane 9 superfamily member 2	4.0	5.9	3	gi|51858639
Heat shock 90 kDa protein 1β	4.7	9.9	5	gi|51859516
Transmembrane 9 superfamily member 4	2.6	5.1	2	gi|68533829
Intercellular adhesion molecule 1	2.4	5.1	2	gi|51980504
Annexin 1 (Lipocortin I)	2.3	13.3	3	gi|6978501
MHC class II antigen	2.0	4.9	1	gi|54780904
Lectin, mannose-binding, 1	2.0	2.1	1	gi|16758758
5′-Nucleotidase	2.0	2.6	1	gi|71051684
Lectin, galactoside-binding, 3 binding protein	2.0	3.1	1	gi|51859422

[a] The score (*S*) is a measure of the confidence in the assignment. Specifically, the relative certainty is $100 \times (1 - 10^{-S})$ percent.
[b] Percent sequence coverage.
[c] Number of sequence-distinct peptides whose assignment contributed to protein's score.
[d] NCBI Entrez protein accession number.

Chinese [19] patients. Hsp 27 has also been discussed as a serum biomarker for hepatocellular carcinoma [35]. Interestingly, the Hsp 27 chaperone was not found in our analyses as a protein that is differentially overexpressed in Morris hepatoma 7777. We have, however, detected this protein in a highly purified membrane fraction of the hepatocellular carcinoma cell line 253T-NT-V [32].

Members of the annexin family are another group of proteins, identified in the pH11- and TX100-solubilizates of Morris hepatoma 7777 (cf. Tables 3 and 4), that we did not identify in the normal liver fractions. Annexins A1, A2, A4, A5, A7 and A11 were identified either in the pH11- or in the TX 100-solubilizate, or in both (cf. Tables 3 and 4). Interestingly, only annexin A6 was identified in both liver and Morris hepatoma 7777 plasma membrane fractions (cf. electronic supplements). We recently found that the low molecular weight annexins are also present in the detergent-insoluble fraction of Morris hepatoma 7777 [11]. Although annexin A6 was identified in the detergent-insoluble fraction of liver plasma membranes, the other annexins were undetected in this fraction. Of all annexins with lower molecular weight, only annexin A3 and annexin A11 were not detected [11]. We have also found annexin A1 and annexin A7 in the highly enriched membrane fractions of the hepatocellular carcinoma cell line 253T-NT-V [32]. Using metal affinity chromatography and 2D electrophoresis prior to mass spectrometry, She et al. [36] identified annexin A2, annexin A4 and annexin A5, together with some members of the heat shock 70 protein family, as being proteins that are differentially overexpressed in hepatocellular carcinomas.

As shown in Table 3, CaBP1 and S100 calcium binding protein A10 are additional calcium binding proteins found in the pH11-extract from Morris hepatoma 7777 [37,38]. S100 calcium binding protein A10 is usually complexed with annexins. These complexes are involved in several cellular events, e.g. exocytosis [38]. Basigin 2 is complexed with other integral membrane proteins such as integrins [39]. Two cancer markers, α-fetoprotein and glutathione *S*-transferase π, are also found in this membrane extract from Morris hepatoma 7777, but not in the corresponding extract from normal liver (cf. Table 3). These proteins may be complexed with other integral or membrane-associated proteins. Another possible explanation for their detection in this extract is that they are highly overexpressed in the hepatoma, and were carried over due to insufficient removal during the first extraction step. Aldolase A, α-fetoprotein and glutathione *S*-transferase are also detected as differentially expressed in the TX100-solubilizate from Morris hepatoma 7777 plasma membranes (cf. Table 4). We also repeatedly found members of the peroxiredoxin family in plasma membrane fractions of hepatocellular carcinomas [11,32]. Peroxiredoxin-3, identified in our TX100 extracts of Morris hepatoma 7777 plasma membranes, is a mitochondrial protein that protects cells against apoptosis caused by oxidizing agents [40].

Transferrin receptor was also identified in both the pH11-solubilizate and the TX100- solubilizate from Morris hepatoma 7777. This membrane protein could not identified in normal liver plasma membranes. Overexpression of transferrin receptor has been repeatedly found in hepatocellular carcinomas, e.g. by genomic methods or by immunohistochemistry [41,42]. Park et al. [43] found overexpression of the transferrin receptor in some hepatocellular carcinomas by use of proteomic methods, 2D-electrophoresis and MALDI-MS. Transferrin receptor is predominantly an integral membrane protein. However, a soluble isoform of this protein also exists [44]. This observation explains the detection of this protein in both membrane-bound (TX100) and membrane-associated (pH11) protein fractions (cf. Tables 3 and 4).

Cadherin is another integral membrane protein identified in the TX100-solubilizate of Morris hepatoma 7777 plasma membranes. Again, this protein was not found in the TX100-solubilizate of the liver plasma membranes. Partnering with catenin, cadherin forms cell–cell adhesion junctions [22-24]. Cadherin was also identified in the F/T-solubilizate from Morris hepatoma 7777 plasma membranes (cf. above and Table 4). The remaining proteins listed in Table 4 are glucosyl transferase [45], two members of the transmembrane 9 protein superfamily [46], MHC class II antigen [47], intercellular adhesion molecule ICAM-1 [48], the integral membrane protein 5′-nucleotidase [49], and two lectins, galactose-binding 3 [50] and mannose-binding 1 [46]. In certain cancers, 5′-nucleotidase is overexpressed and released into serum [51]. The relevance of finding the other proteins in the TX100 extract from Morris hepatoma 7777, but not in the corresponding extract from liver plasma membranes, is not clear.

Our method for selective solubilization of plasma membranes, and the subsequent identification and direct comparison of the proteins from normal liver and Morris hepatoma 7777, enables identification of proteins that are present in one organ (e.g., liver) but not detectable in the corresponding tissue. Here, we discuss the possibility that proteins detected in the plasma membrane extracts from Morris hepatoma 7777, but not detected in corresponding extracts from liver plasma membranes, might be candidate markers for hepatocellular carcinomas. Some of proteins detected here, e.g. members of the heat shock family, have already been discussed as such biomarkers [19]. We also found that some low molecular weight numbers of the annexin family are present in plasma membrane fractions of Morris hepatomas and one hepatocellular carcinoma cell line [11,32]. These annexin could be not found in corresponding normal liver plasma membrane fractions. By use of histochemical and immunochemical methods, overexpression of low molecular weight annexins, such as annexin A1 and A2, has already been detected in many carcinomas [38,52]. However, only one other group using proteomic methods has detected the overexpression of these proteins in hepatocellular carcinomas [43]. This failure of detect annexins may be due to different analytical methods of protein separation prior to MS. Most MS identifications are performed after 2D-electrophoretic separation of proteins from whole cancer or liver tissue, without pre-fractionation [19,33–35,53–55]. Fractionation of cells into organelles [32] and further fractionation of organellar proteins according to their binding to metal ions [43], their charge [14] or, as presented here, their hydrophobicity, enables further identification of less abundant proteins as candidates for cancer biomarkers. In our ongoing work, we use further fractionation methods, such as isoelectric focussing and

size-exclusion and ion-exchange chromatography to identify additional proteins differentially expressed in liver and Morris hepatoma plasma membranes. The proteins listed in Tables 2–4 were found in hepatoma, but not in liver, plasma membranes. By use of these additional fractionation strategies, we are able to find further candidate biomarkers in this model system (Clifton, Josic et al., to be published).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2006.08.047.

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