Anal. Chem. **2002,** *74,* 1779−1791

A 2-D Liquid Separations/Mass Mapping Method for Interlysate Comparison of Ovarian Cancers

Maureen T. Kachman,[†] Haixing Wang,[†] Donald R. Schwartz,[‡] Kathleen R. Cho,[‡] and David M. Lubman*,[†]

Department of Chemistry, and Department of Pathology, School of Medicine, The University of Michigan, Ann Arbor, Michigan 48109-1055

A two-dimensional liquid phase separation of proteins from whole cell lysates coupled on-line to an electrospray-ionization time-of-flight (ESI-TOF) mass spectrometer (MS) is used to map the protein content of ovarian surface epithelial cells (OSE) and an ovarian carcinoma-derived cell line (ES2). The two dimensions involve the use of liquid isoelectric focusing as the first phase and nonporous silica reversed-phase HPLC as the second phase of separation. Accurate molecular weight (MW) values are then obtained upon the basis of ESI-TOFMS so that an image of isolectric point (p*I*) versus MW analogous to 2-D gel electrophoresis is produced. The accurate MW together with the p*I* fraction and corresponding hydrophobicity (%B) are used to tag each protein so that protein expression can be compared in interlysate studies. Each protein is also identified on the basis of matrix-assisted laser desorption−ionization (MALDI) TOFMS peptide mapping and intact MW so that a standard map is produced against which other cell lines can be compared. Quantitative changes in protein expression are measured in these interlysate comparisons using internal standards in the on-line ESI-TOFMS process. In the ovarian epithelial cell lines under study, it is shown that in the three p*I* fractions chosen for detailed analysis, over 50 unique proteins can be detected per fraction, of which 40% can be identified from web-based databases. It is also shown that when using an accurate MW to compare proteins in the OSE versus ovarian cancer sample, there are proteins highly expressed in cancer cells but not in normal cells. In addition, many of the proteins in the cancer sample appear to be down-regulated, as compared to the normal cells. This two-dimensional (2-D) liquid/mass mapping method may provide a means of studying proteins in interlysate comparisons not readily available by other methods.

An important area of cancer research requires the ability to assess changes in cellular protein expression during cancer progression.[1−10] Cancers are thought to arise through a multistep process in which repeated cycles of somatic mutation and clonal selection produce variant progeny with increasingly aggressive growth properties. The genes mutated in cancer frequently encode proteins that function in conserved signaling pathways, and hence, the mutations result in alterations of the signaling mechanisms within the cancer cells.[11−15] The critical consequence of gene mutations is altered protein expression within cancer cells, which may be reflected in expression of new proteins, differences in the amount of expressed proteins, and differences in posttranslational protein modifications, such as phosphorylation and acetylation. It thus becomes important to be able to monitor protein expression in cancer cells versus normal cells and to use interlysate comparisons to relate protein expression changes to different stages of cancer development. Understanding these protein expression changes during tumorigenesis may lead to mechanistic insights into the neoplastic process as well as the identification of protein markers useful for cancer diagnosis and assessment of prognosis. Numerous previous studies of various types of cancers have related protein expression to the diagnosis of specific forms of cancer and to the prognosis of the disease.[1−6,16−18] Perhaps more

[†] Department of Chemistry.

[‡] Department of Pathology, School of Medicine.

(1) Bergman, A. C.; Benjamin T.; Alaiya A.; Waltham M.; Sakaguchi K.; Franzen B.; Linder S.; Bergman T.; Auer G.; Appella E.; Wirth P. J.; Jornvall, H. *Electrophoresis* **2000**, *21*, 679−686.

(2) Alaiya, A. A.; Franzen, B.; Auer, G.; Linder, S. *Electrophoresis* **2000**, *21* (6), 1210−1217.

(3) Kellner, R. *Fresenius' J. Anal. Chem.* **2000**, *366* (6−7), 517−524.

(4) Giometti, C. S.; Williams, K.; Tollaksen, S. L. *Electrophoresis* **1997**, *18* (3−4), 573−581.

(5) Stulik, J.; Hernychova, L.; Porkertova, S.; Knizek, J.; Macela, A.; Bures, J.; Jandik, P.; Langridge, J. I.; Jungblut, P. R. *Electrophoresis* **2001**, *22*, 3019−3025.

(6) Seow, T. K.; Ong, S.; Liang, R. C. M.; Ren, E.; Chan, L.; Ou, K.; Chung, M. C. M. *Electrophoresis* **2000**, *21*, 1787−1813.

(7) In *Proteome Research: New Frontiers in Functional Genomics*; Wilkens, M. R., Williams, K. L., Appel, R. D., Hochstrasser, D. F., Eds.; Springer: Berlin, 1997; pp 187−212.

(8) Masumori, N.; Thomas, T. Z.; Chaurand, P.; Case, T.; Paul, M.; Kasper, S.; Caprioli, R. M.; Tsukamoto, T.; Shappell, S. B.; Matusik, R. J. *Cancer Res.* **2001**, *61* (5), 2239−2249.

(9) Stoeckli, M.; Chaurand, P.; Hallahan, D. E.; Caprioli, R. M. *Nat. Med.* **2001**, *7* (4), 493−496.

(10) Chong, B. E.; Hamler, R. L.; Lubman, D. M.; Ethier, S. P.; Rosenspire, A. J.; Miller, F. R. *Anal. Chem.* **2001**, *73*, 1219−1227.

(11) Minamoto, T.; Buschmann, T.; Habelhah, H.; Matusevich, E.; Tahara, H.; Boerresen-Dale, A.; Harris, C.; Sidransky, D.; Ronai, Z. *Oncogene* **2001**, *20*, 3341−3347.

(12) Egan, C.; Pang, A.; Durda, D.; Cheng, H. C.; Wang, J. H.; Fujita, D. J. *Oncogene* **1999**, *18*, 1227−1237.

(13) Muller, D. R.; Schindler, P.; Coulot, H.; Veshol, H.; van Oostrum, J. *J. Mass Spectrom.* **1999**, *34* (4), 336−345.

(14) Zugaro, L. M.; Reid, G. E.; Ji, H.; Eddes, J. S.; Murphy, A. C.; Burgess, A. W.; Simpson, R. J. *Electrophoresis* **1998**, *19* (5), 867−876.

(15) Godovac-Zimmerman, J.; Soskic, V.; Poznanovic, S.; Brianza, F. *Electrophoresis* **1999**, *20*, 952−961.

10.1021/ac011159c CCC: $22.00
Published on Web 03/14/2002

importantly, such studies may also lead to the identification of proteins that may serve as novel drug targets for tumor-specific treatment.

The method currently used to compare protein expression profiles of different samples is two-dimensional (2-D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE).[19−22] The 2-D gel method has proven to be a modestly successful means of imaging a large number of proteins within a given sample and allowing sample-to-sample comparison of protein expression profiles. Researchers can search for differences in either MW or p*I* shifts related to changes in structure or to quantitative differences in protein expression. In practice, however, it has often proven difficult to make such comparisons, largely due to poor reproducibility of conditions for running and staining gels. Variability of gel conditions can lead to shifts in protein spot positions and difficulties making quantitative comparisons between spots. More recent studies have attempted to improve such comparisons by labeling proteins from one cell lysate with one color dye and labeling the second lysate with a second color dye.[23] If both lysates are run on a gel together, then accurate comparisons can be made using differential color display. However, this works only on two samples run simultaneously and still lacks a standard method for identifying and comparing proteins between large numbers of cell lysates.

In this work, we describe an alternative method to 2-D gels for accurate interlysate comparisons. The method for protein separation has been introduced in previous work based upon a 2-D liquid-phase separation using liquid-phase isoelectric focusing (IEF) in the first dimension and nonporous silica (NPS) reversed-phase (RP)-HPLC in the second dimension.[24,25] A number of other liquid-based methods have also been recently introduced for screening the protein content of cells.[26−39] An important advantage of the method presented herein is that the liquid eluent of RP-HPLC can be directed into an ESI-TOFMS for accurate intact MW analysis. The result is a 2-D virtual image of p*I* vs MW analogous to 2-D gels,[40] but in which a MW accurate to <150 parts per million (ppm) can be obtained. The key issue for cancer studies is that reproducible interlysate comparisons can be performed using the intact MW of each protein determined by mass spectrometry. This method avoids the problem of poor reproducibility of electrophoretic separation conditions on 2-D gels. Moreover, we show that the use of the 2-D liquid phase separation allows rapid collection and identification of large numbers of proteins from cancer cells. The result is a standard 2-D mass map with identifications associated with many of the bands, which becomes important in statistical analysis of protein expression changes associated with disease progression. In the present study, we generated standard 2-D mass maps of cultured normal ovarian surface epithelium cells and an epithelial ovarian cancer-derived cell line in order to identify proteins that are differentially expressed, which may be important in the cancer process. The reproducibility of the 2-D liquid separations/mass mapping technique has the potential to yield essential information related to cancer and other human diseases on the basis of sample-to-sample comparison of protein expression profiles.

EXPERIMENTAL SECTION

Cell Culture and Sample Preparation. The ovarian clear cell adenocarcinoma cell line ES2 was obtained from the American Type Tissue Culture Collection (Manassas, VA). HPV16 E6/E7 immortalized ovarian surface epithelial (OSE) cell lines 96.9.18 and 96.1.24 were obtained from Dr. Wayne Lancaster (Wayne State University, Detroit, MI). Life-extended (with SV-40 large T antigen) OSE cell lines IOSE-144 and IOSE-80 were obtained from Dr. Nelly Auersperg (University of British Columbia, Vancouver, Canada). Unless stated otherwise, all media and media supplements were obtained from Life Technologies, Inc. (Gaithersburg, MD), and all cells were cultured using standard cell culture techniques. OSE were cultured in DMEM, and ES2 was grown in McCoy's 5A medium. All media were supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 mg/mL streptomycin. Cultured cells (80−90% confluent monolayer) were washed three times with phosphate buffered saline (PBS), and the residual PBS from the final wash was completely removed by aspiration. Solubilization solution [1% *n*-octyl β-D-glucopyranoside (OGl) (Sigma); 6 M urea (ICN); 2 M thiourea (ICN); 10 mM dTT (Sigma); and 2.5% Biolyte ampholytes, pH 3.5−10 (Biorad); and 10 mM PMSF] was added (300 μL/T-75 flask), and the cells were removed with a cell scraper. An additional 200 μL of solubilization solution was used to remove any residual cell lysate. Insoluble

(16) Ji, H.; Reid, G. E.; Moritz, R. L.; Eddes, J. S.; Burgess, A. W.; Simpson, R. J. *Electrophoresis* **1997**, *18*, 605−613.
(17) Rasmussen, H. H.; Orntoft, T. F.; Wolf, H.; Celis, J. E. *J. Urol.* **1996**, *155* (6), 2113−2119.
(18) Schwalke, M. A.; Doremus, C. M.; Bleday, R.; Wanebo, H. J.; Vezeridis, W. P. *Arch. Surg.* **1990**, *125*, 469−471.
(19) O'Farrell, P. H. *J. Biol. Chem.* **1975**, *250*, 4007−4021.
(20) Gorg, A.; Obermaier, C.; Boguth, G.; Harder, A.; Scheibe, B.; Wildgruber, R.; Weiss, W. *Electrophoresis* **2000**, *21*, 1037−1053.
(21) *Advances in Electrophoresis*; Chrambach, A., Dunn, M. J., Radola, B. J., Eds,; VCH: Weinhein, 1987; pp 1−109.
(22) *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods*; Rabilloud, T., Ed.; Springer-Verlag: Berlin, 2000.
(23) Unlu, M.; Morgan, M. E.; Minden, J. S. *Electrophoresis* **1997**, *18* (11), 2071−2077.
(24) Wall D. B.; Kachman M. T.; Gong S.; Hinderer, R.; Parus S. J.; Misek, D. E.; Hanash, S. M.; Lubman, D. M. *Anal. Chem.* **2000**, *72*, 1099−1111.
(25) Wall, D. B.; Kachman M. T.; Gong S.; Parus, S. J.; Long, M. W.; Lubman D. M. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1649−1661.
(26) Nilsson, C. L.; Larsson, T.; Gustafsson, E.; Karlsson, K.; Davidsson, P. *Anal. Chem.* **2000**, *72*, 2148−2153.
(27) Nilsson, C. L.; Purchades, M.; Westman, A.; Blennow, K.; Davidson, P. *Electrophoresis* **1999**, *20* (4−5), 860−865.
(28) Jensen, P. J.; Pasa-Tolic, L.; Anderson, G. A.; Horner, J. A.; Lipton, M. S.; Bruce, J. E.; Smith, R. D. *Anal. Chem.* **1999**, *71*, 2076−2084.
(29) Opitek, G. J.; Ramirez, S. M.; Jorgenson, J. W.; Moseley, M. A. *Anal. Biochem.* **1998**, *258*, 349−361.
(30) Udiaver, S.; Apffel, A.; Chakel, J.; Swedberg, S.; Hancock, W. S.; Pungor, W. S. *Anal. Chem.* **1998**, *70*, 3572−3578.
(31) Hoffmann, P.; Ji, H.; Moritz, R. L.; Connolly, L. M.; Frecklington, D. F.; Layton, M. J.; Eddes, J. S.; Simpson, R. J. *Proteomics* **2001**, *1*, 807−818.
(32) Dai, Y.; Li, L.; Rosen, D. C.; Long, S. R. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 73−78.
(33) Wagner, K.; Racaityte, K.; Unger, K. K.; Miliotis, T.; Edholm, L. E.; Bischoff, R.; Marko-Varga, G. *J. Chromatogr. A* **2000**, *893* (2), 293−305.
(34) Zuo, X.; Speicher, D. W. *Anal. Biochem.* **2000**, *284* (2), 266−278.
(35) Premstaller, A.; Oberacher, H.; Walcher, W.; Timperio, A. M.; Zolla, L.; Chervet, J. P.; Cavusoglu, N.; van Dorsselaer, A.; Huber, C. G. *Anal. Chem.* **2001**, *73* (11), 2390.
(36) Burton, W. G.; Nugent, K. D.; Slattery, T. K.; Summers, B. R.; Snyder, L. R. *J. Chromatogr.* **1988**, *443*, 363−379
(37) Raida, M.; Schulz-Knappe, P.; Heine, G.; Forssmann, W. G. *J. Am. Soc. Mass Spectrom.* **1999**, *10* (1), 45−54.
(38) Hille, J. M.; Freed, A. L.; Watzig, H. *Electrophoresis* **2001**, *22*, 4035−4052.
(39) Hutchens, T. W.; Gibbons, W. E.; Besch, P. K. *J. Chromatogr.* **1984**, *297*, 283−299.
(40) Walker, A. K.; Rymar, G.; Andrews, P. C. *Electrophoresis* **2001**, *22* (5), 933−945.

Table 1. Proteins Identified in ES2 and OSE with Quantification and Hydrophobicity Comparison

figure label	protein name	accession no.	MALDI % coverage	MS-Fit MW	OSE LCT MW	% B	ES2 LCT MW	% B	% change in expression ES2 vs OSE
6a	HSP71	P11142	39	70 898	70 890	44.54	70 891	44.34	174
6b	DNA polymerase ϵ subunit B	P56282	16	59 537			59 610	48.65	*a*
6c	acetylcholine receptor protein, β chain precursor	P11320	17	56 726			56 732	50.38	*a*
6d	vimentin	P08670	39	53 686	53 586	41.71	53 580	41.68	90
6e	keratin, type II cytoskeletal 8	P05787	20	53 674	53 643	44.69	53 656	44.73	123
6f	keratin, type II cytoskeletal 7 (cytokeratin 7)	P08729	35	51 335	51 336	43.11	51 337	43.09	62
6g	telomeric repeat binding factor 1	P54274	26	50 345	50 363	43.08	50 359	42.91	33
6h	tubulin α-1 chain, brain-specific	P04687	33	50158	50 161	46.20	50 165	46.03	159
6i	tubulin α-4 chain	P05215	28	49 924	49 917	46.20	49 917	46.03	187
6j	tubulin β-1 chain, actually TBB	P07437	31	49 758	49 690	47.21	49 687	47.08	115
6k	keratin, type I cytoskeletal 18	P05783	19	48 058	48 046	46.83	48 033	46.88	−59
6l	KIAA0513	O60268	18	46 639			46 660	46.16	*a*
6m	eukaryotic initiation factor 4A-I	P04765	31	46 154	46 084	50.50	46 084	50.48	214
6n	actin, cytoplasmic 2 (γ-actin)	P02571	46	41 793	41 724	48.20	41 729	48.01	10
6o	actin, cytoplasmic 1 (β-actin)	P02570	39	41 737	41 677	48.20	41 677	48.01	109
6p	HLA class I histocompatibility antigen α chain precursor	P30498	24	40 478	40 347	41.80	40 337	41.50	−2
6q	hydroxyindole *o*-methyltransferase	P46597	22	38 453	38 324	39.04			*a*
6r	inorganic pyrophosphatase	Q15181	31	32 660	32 586	40.49	32 588	40.39	2212
6s	chloride intracellular channel protein 1	O00299	41	26 923	26846	45.46	26 847	45.23	139
6t	ubiquitin carboxyl-terminal hydrolase isozyme L1	P09936	24	24 824	24 834	41.93	24 834	41.60	37
6u	RAN-specific GTPase-activating protein	P43487	49	23 310	23 232	37.42	23 233	37.24	233
6v	peroxiredoxin 2 (thioredoxin	*P*32119	26	21 892	21 810	44.68	21 814	44.73	−27
6w	ATP synthase D chain, mitochondrial	O75947	43	18 491	18 409	40.23	18 415	39.86	182
6x	GLIA maturation factor β (GMF-β)	P17774	27	16 713	16 838	44.21	16 839	44.14	76
			Fraction 7						
7a	mitochondrial stress-70 protein precursor	P38646	17	73 780.3	73 812	50.33	73 780	50.45	−351
7b	T-plastin	913797	31	70 436	70 388	49.35	70 377	49.58	121
7c	fragile X mental retardation syndrome related protein 1	P51114	33	69 692.3			69 778	43.09	*a*
7d	ezrin (p81) (cytovillin) (villin 2)	P15311	18	69 399.4	69 308	42.51			*a*
7e	T-complex protein 1, ϵ subunit	P48643	29	59 621	59 627	49.56	59 605	49.60	195
7f	protein disulfide isomerase A3 precursor	P30101	21	56 782.9	56 782	42.16	56 782	42.43	−89
7g	keratin, type II cytoskeletal 8	P05787	31	53 674	53 643	45.88	53 643	45.51	
7h	glutathione synthetase	P48637	20	52 385.3	52 311	50.01	52 312	50.10	88
7i	P59 protein	Q02790	28	51 805	51 732	39.56	51 739	39.73	−12
7j	keratin, type II cytoskeletal 7	P08729	26	51 335	51 326	44.41	51 320	44.43	
7k	RAB GDP dissociation inhibitor α	P31150	23	50 583.2	50 721	46.83	50 735	47.10	82
7l	tubulin α-1 chain, brain-specific	P04687	29	50 158	50 168	46.83	50 179	47.10	
7m	probable ATP-dependent RNA helicase P47	Q13838	30	48 991	48 913	49.01	48 921	49.11	272
7n	actin-like protein 3	P32391	22	47 371	47 315	48.65	47 300	48.76	111
7o	actin, cytoplasmic 2 (γ-actin)	P02571	27	41 793	41 677	48.65	41 674	48.76	
7p	actin, cytoplasmic 1 (β-actin)	P02570	30	41 737	41 735	48.65	41 736	48.76	
7q	L-lactate dehydrogenase H chain (LDH-B)	P07195	12	36 638.8	36 561	50.33	36 566	50.35	296
7r	inorganic pyrophosphatase	Q15181	31	32 660	32 714	39.56	32 712	39.79	578
7s	B23 nucleophosmin	X16934	18	30 938.4	30 887	39.56	30 906	39.79	144
7t	cytokine-inducible SH2-containing protein	Q9NSE2	31	28 663.2	28 645	49.01	28 644	49.16	−59
7u	(TPI1.)triosephosphate isomerase (EC 5.3.1.1) (TIM)	P00938	17	26 538.5	26 584	45.26	26 584	45.43	82
7v	glutathione *s*-transferase P	P09211	25	23 356	23 232	44.51	23 221	44.81	81
7w	heat shock 27 kda protein (HSP 27)	P04792	25	22 782	22 785	38.62	22 782	38.87	−261
7x	interferon α-1/13 precursor	P01562	36	21 725.3	21 810	45.21	21 812	45.43	−71
7y	nucleoside diphosphate kinase A (NDK A) (NDP kinase A)	P15531	37	17 148.9	17 073	43.64	17 067	43.49	127
			Fraction 14						
14a	pyruvate kinase, M1	P14618	34	57 858	57 861	44.78	57 851	44.59	109
14b	intercellular adhesion molecule-1 precursor (ICAM-1)	P05362	16	57 826	57 826	52.48	57 818	52.40	268
14c	dyskerin (nucleolar protein NAP57)	O60832	18	57 674.6			57 667	43.20	*a*

Table 1 (Continued)

figure label	protein name	accession no.	MALDI % coverage	MS-Fit MW	OSE LCT MW	% B	ES2 LCT MW	% B	% change in expression ES2 vs OSE
			Fraction 14						
14d	GTP-binding protein ERA homolog	O75616	22	49 098.2	49 231	49.40	49 207	49.43	74
14e	α-enolse	P06733	17	47 037	47 093	43.73	47 083	44.05	142
14f	collagen-binding protein 2 precursor (colligin 2)	P50454	44	46 536.1	46 509	42.09	46 511	42.23	193
14g	47 KDA heat shock protein precursor (colligin 1)	P29043	37	46 267.7	46 271	42.09			*a*
14h	*β*-1,4-galactosyltransferase 6	Q9UBX8	17	44 914	44 926	50.11			*a*
14i	phosphoglycerate kinase 1	P00558	29	44 728	44 547	49.66	44 527	49.58	373
14j	fructose-bisphosphate aldolase A	P04075	25	39 420	39 298	42.34	39 290	42.29	324
14k	annexin II	P07355	33	38 604	38 525	46.63	38 533	46.46	122
14l	L-lactate dehydrogenase M chain (LDH-A)	P00338	29	36 689	36 608	50.11	36 600	50.01	204
14m	hnRNP A2 protein	337449	33	36 006.3	36 076	37.49	36 098	37.49	14
14n	glyceraldehyde 3-phosphate dehydrogenase	P04406	28	35 922.02	35 931	43.08	35 923	43.03	77
14o	brain-derived neurotrophic factor precursor (BDNF)	*P*23560	25	27 818.2	27 813	45.74	27 798	45.71	9
14p	PPIase	P05092	37	18 012	18 061	39.82	18 057	39.76	118
14q	nucleoside diphosphate kinase B	P22392	41	17 298	17 220	42.73	17 207	42.81	56
14r	putative RNA-binding protein 3	P98179	31	17 170.5	17 174	37.49	17 171	37.49	88
14s	profilin	P07737	50	15 054.4	15 207	37.89	15 201	37.74	1

[a] Percent change >10 000.

material was removed by centrifugation (10000*g*, 10 min, 4 °C), and the supernatant was stored at −80 °C until further use. Using this procedure, both cytosolic and membrane proteins were available for analysis.[24,26] Equal amounts of protein (2.5 mg) from each of the four OSE cell lines were pooled prior to Rotofor isoelectric focusing.

Rotofor Isoelectric Focusing. A preparative-scale Rotofor[41] was used in the first-dimension separation. Approximately 10 mg of protein from the whole cell lysates was diluted to a final volume of ∼55 mL with the Rotofor running buffer consisting of 0.1% OG (Sigma), 8 M urea (ICN), 2 M thiourea (ICN), 10 mM DTT (Sigma), and 2.5% Biolyte ampholytes (Biorad). The procedure used for running the Rotofor was a modified version of the standard procedure described in the Rotofor operations manual. After focusing the proteins for 4.5 h, 20 fractions were collected simultaneously into separate vials, and these were divided into 400-μL aliquots and stored at −80 °C until further use. The pH of each fraction was determined using a micro pH meter (PH/C 900 Amersham Pharmacia Biotech). Fractions were quantified on a UV−vis spectrophotometer at 595 nm using the Protein Quantitation Kit I (Biorad) with the BSA standard.

NPS RP-HPLC/LC-MS. Separations were performed at a flow rate of 0.2 mL/min on an analytical (3.0 × 53 mm) NPS RP-HPLC column containing 1.5-μm C18 (ODSI) nonporous beads (Eichrom Technologies).[42−45] The column was placed in a column heater (Timberline, Boulder, CO) and held at 65 °C. The separations were performed using a water/acetonitrile gradient (0.1% trifluoroacetic acid TFA, 0.3% formic acid). The acetonitrile gradient profile was as follows: 10−25% for 2 min, 25−35% for 5 min, 35−45% for 10 min, 45−75% for 10 min and 75−100% for 1 min. Acetonitrile was HPLC grade (99.93+%, Sigma), TFA was from 1-mL sealed ampules (Sigma), and formic acid was ACS grade (Sigma). Water was purified using the Millipore RG system. The HPLC system was a Beckman model 127s/166, and peaks were detected on-line using a commercial ESI-TOF/MS system (LCT, Micromass, Manchester, U.K.).

ESI-TOFMS of NPS HPLC Separated Proteins. The ESI-TOFMS analyses were performed on a LCT as previously described,[25] except that the desolvation temperature was held at 350 °C, and the desolvation gas was held at 575 L/hr. Between 40 and 60 μg of total protein for each Rotofor fraction was injected onto the LCT for the creation of the 2-D p*I*/MW maps. The difference in total protein injected between any two cancer and normal Rotofor fractions was held to <15%. Prior to injection of sample into the LCT, 0.2 μg of bovine insulin (Sigma) was added to the fraction for calibration and quantification purposes. Bovine insulin eluted from the HPLC column prior to any sample proteins. Prior to each run, horse heart myoglobin (Sigma) was directly injected into the mass spectrometer for external calibration. After each run, the 1912.197 peak of bovine insulin was used as a lock mass to fine-tune the external calibration.

Micromass' MassLynx v 3.4 and MaxEnt (version 1) software were used for data analysis. The TIC was scanned for regions that contained redundant multiply charged peaks, and those regions were combined for deconvolution. Deconvolution was performed using a target mass range of 5−85 kDa, 1 Da resolution, 0.75 Da peak width, and a 65% peak height value. The deconvoluted peaks were then combined into a single mass spectrum for each TIC. The combined mass spectrum was converted to a

(41) Petrach, J. M., *Electrophoresis* **1991**, *12* (1), 84−90.
(42) Itoh, H.; Nimura, N.; Kinoshita, T.; Nagae, N.; Nomura, M. *Anal. Biochem.* **1991**, *199*, 7−10.
(43) Banks, J. F.; Gulcicek, E. E. *Anal. Chem.* **1997**, *69*, 3973−3978.
(44) Barder, T. J.; Wohlman, P. J.; Thrall, C.; DuBois, P. D. *LC−GC* **1997**, *15*, 918−926.
(45) Hanson, M.; Unger, K. K.; Mant, C. T.; Hodges, R. S. *Trends Anal. Chem.* **1996**, *15*, 102−110.

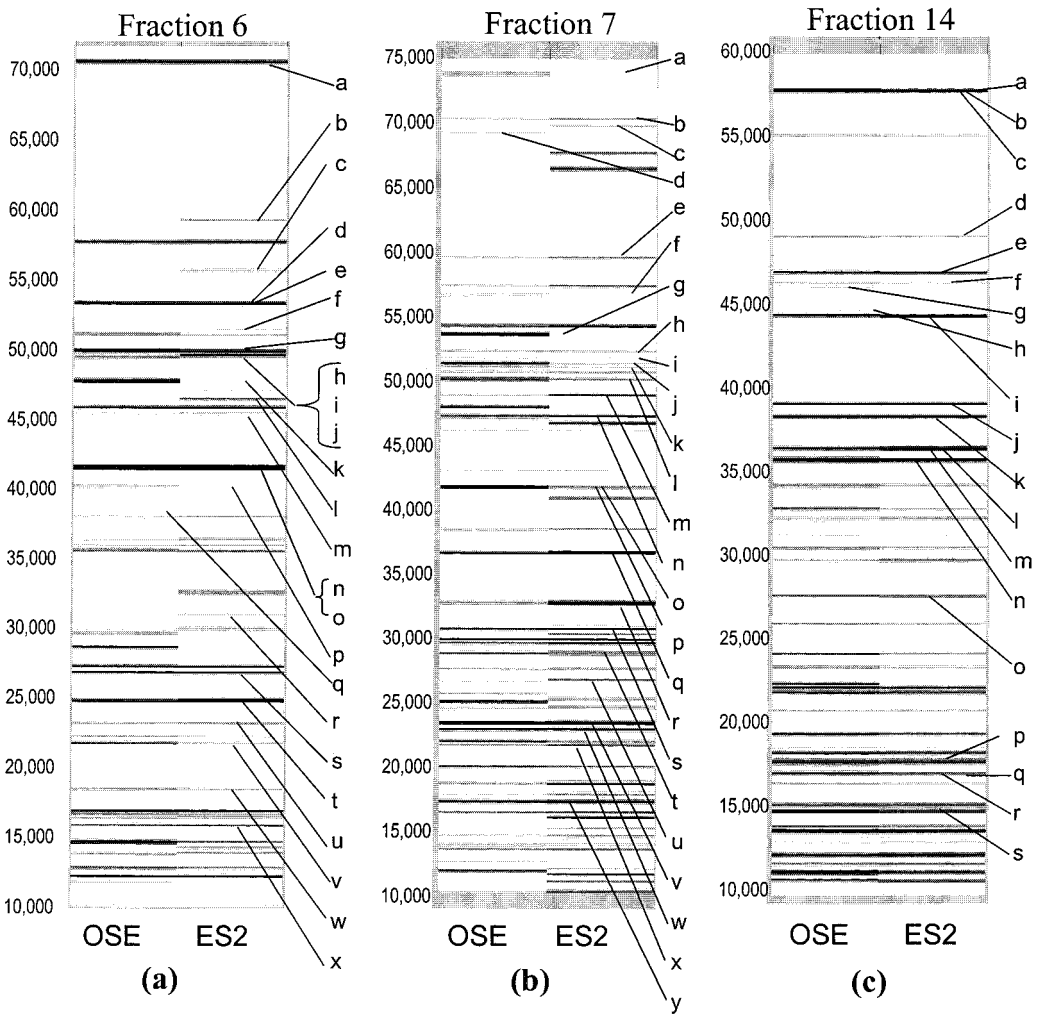

Figure 1. 2-Dimensional mass maps of MW versus p*I* comparing the ES2 cell line to the OSE cell line for Rotofor fraction nos. (a) 6, (b) 7, and (c) 14. The names of proteins identified by MALDI-TOFMS peptide mapping are listed with the corresponding MW bands according to the labeling scheme of Table 1.

text file for input into the 2-D mapping software and the differential display software that were developed in-house. The combined mass spectrum for each OSE fraction was multiplied by a quantity factor, which was the quantity of protein injected into the ES2 LCT run divided by the quantity injected for the OSE run of the same fraction number. The combined mass spectrum for each OSE fraction was also multiplied by an ionization factor to account for the ionization efficiency of the LCT for that run. The ionization factor was the intensity of the bovine insulin standard detected in the ES2 LCT run divided by the OSE run for a particular fraction number.

NPS RP-HPLC Separations with Fraction Collection. Separations were performed at a flow rate of 1.0 mL/min on an 8.0 × 33 mm NPS RP-HPLC column containing 1.5 μm C18 (ODSI) nonporous beads (Eichrom Technologies.) The column was placed in a column heater (Timberline, Boulder, CO) and held at 65 °C. The separations were performed using a water/acetonitrile gradient containing 0.1% TFA and 0.05% *n*-octyl β-D-glucopyranoside (OGl, Sigma). The gradient profile was as follows: 10−25% for 2 min, 25−35% for 5 min, 35−45% for 10 min, 45−75% for 10 min, and 75−100% for 1 min. Acetonitrile was HPLC grade (99.93+%, Sigma), TFA was from 1-mL sealed ampules (Sigma), and formic acid was ACS grade (Sigma). Water was purified using the Millipore RG system. Proteins were collected as fractions into 1.5-mL polypropylene tubes using a semiautomated in-house-designed fraction collection system.

A random sampling of collected protein peaks were reinjected via syringe pump into the LCT in order to compare retention times between the on-line and the off-line HPLC systems. From these data (not shown), a delay of 2.0 min for the on-line system, as compared with the off-line system, was established.

Enzymatic Digestion of Protein Fractions. Collected fractions were dried to 50% of their original volume to remove the acetonitrile and TFA, followed by the addition of 10% (v/v) 10 mM DTT, 10% (v/v) 1 M NH_4HCO_3, and 0.25 μg of TPCK-treated trypsin (Promega). The fractions were then incubated at 37 °C for 24 h. After 24 h, 2.5% (v/v) TFA was added to stop digestion. Fractions were stored at 4 °C until further analysis.

MALDI-MS Analysis of Protein Digests. Prior to MALDI analysis, the proteins were purified and desalted using 2-μm C_{18} ZipTips (Millipore) and a final elution volume of 10 μL. A 0.5-μL portion of purified protein solution was spotted into each well of a 100-well MALDI plate (Perseptive Biosystems). For internal calibration, 50 fmol of angiotensin I, adrenocorticotropin (ACTH, amino acids 1−17), ACTH(18−39), and ACTH(7−38) were added to a 1:4 dilution of saturated α-cyano-4-hydroxycin-

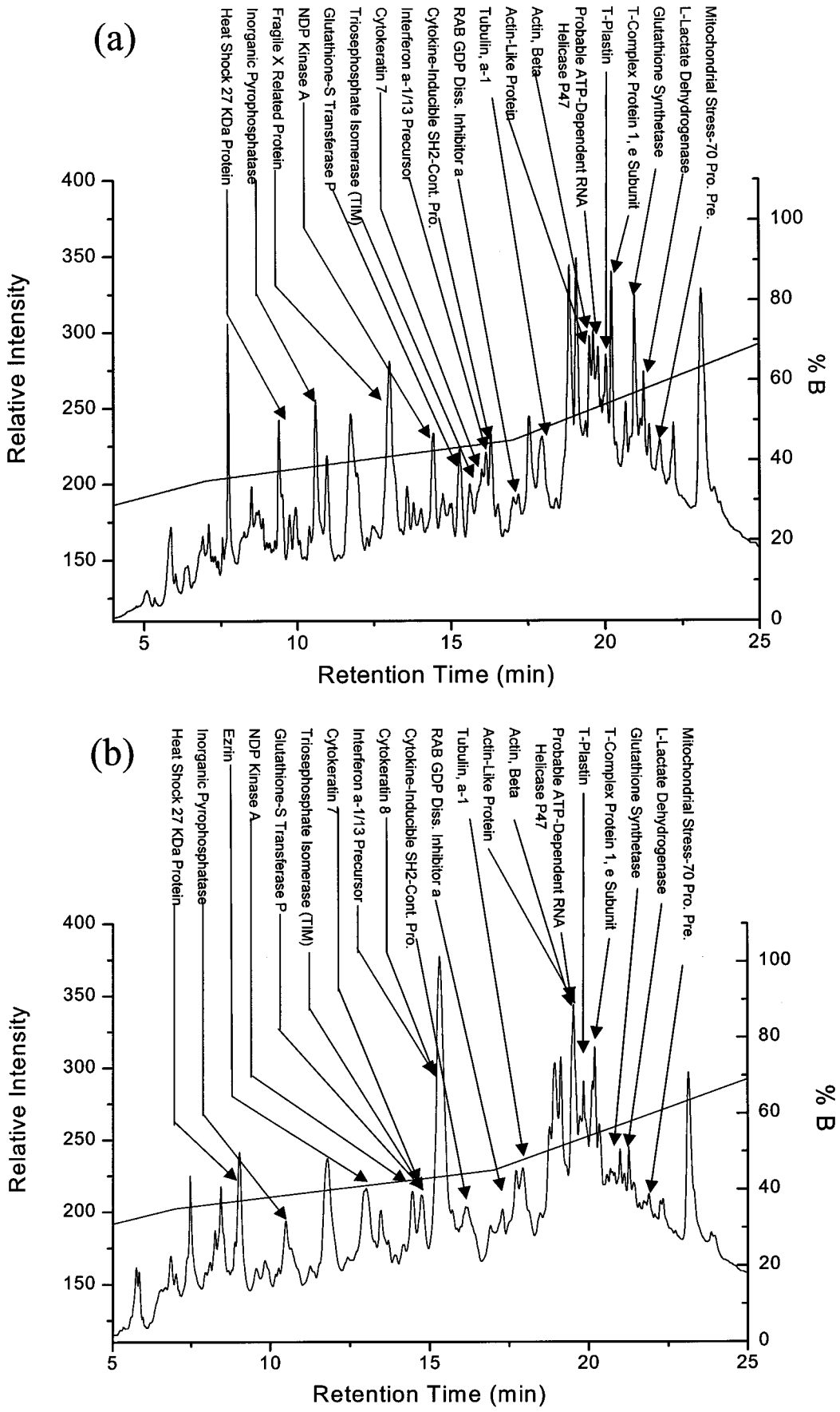

Figure 2. NPS RP-HPLC chromatogram of Rotofor fraction 7 for (a) ES2 cell line and (b) OSE cell line with detection by UV absorption at 214 nm. The names of proteins identified by liquid fraction collection, tryptic digestion, and MALDI-TOFMS peptide mapping are listed with the corresponding chromatographic peak.

namic acid (α-CHCA, in 50% ACN, 1% TFA), and 0.5 μL of this mixture was added on top of the sample before the sample was dried. MALDI-MS was performed using a delayed extraction reflectron-equipped MALDI-TOFMS instrument (STR, Perseptive). The repeller voltage was set at +25 kV, the grid voltage was at 72% of repeller voltage, the delay time was 100 ns, and the reflectron was set to a ratio of 1.12. Each peptide mass spectrum was the average of 100−150 spectra.

The resolution of the Voyager-STR MALDI-TOFMS was >10 000 Da for the entire peptide mass range from 800 to 4000 Da. Using internal calibration standards as described above, a mass accuracy of 20 ppm was typically achieved for peptide masses from 800 to 4000 Da. Monoisotopic peptide masses were submitted to either MS-Fit (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) or Peptident (http://www.expasy.ch/tools/peptident.html) for identification. A "strong" hit (an identification with high confidence) met the following criteria: (1) The protein was ranked first or second by the search engine. (2) The percent coverage was at least 20% for large proteins(>30 kDa) and >20% for smaller proteins. (3) The average mass error was small (20 ppm). (4) The matched peptides included the most abundant peaks in the MALDI spectrum. In addition, most of the proteins we identified satisfied additional criteria, although these criteria would not be used to disallow a match due to the possibility of p*I* or mass shifts resulting from posttranslational modification(s). The additional criteria include: (1) The reported (from the search engine) p*I* was within 0.5 units of the Rotofor fraction p*I* in which the protein was found. (2) The MW of the protein in the database was expected to fall within 300 ppm of the mass detected by the LCT.

RESULTS AND DISCUSSION

Figure 1 shows, in a differential format, a comparison of the 2-D liquid separation/mass maps of an ovarian carcinoma-derived cell line versus the pooled OSE. The image is displayed in a format analogous to 2-D gels with p*I* on the *x* axis and MW on the *y* axis. In this figure, each of three different Rotofor fractions, 6, 7, and 14, of p*I* values 5.9, 6.4, and 8.4 are directly compared between the ES2 ovarian cancer cell line and the pool of four OSE cell lines. The resolution in the p*I* axis is 0.5 pH units; in the MW axis, resolution is 5000. The graphical format does not accurately show the resolution on the *y* axis, although this can be recovered by clicking on the bands in the image to obtain the deconvoluted mass spectrum. In addition, each protein band has been collected and digested by trypsin and analyzed by MALDI-TOFMS peptide mapping. The identity of each band is indicated for each protein for which there was a strong positive identification on the basis of database searching. The proteins are labeled according to the scheme corresponding to that used in Table 1. Additionally, Table 1 shows the intact MW for proteins from both the pooled OSE and ES2 cell lines and these are compared to the database MW reported by Msfit. In addition, changes in quantitative expression of proteins in the two cell lines are shown.

The protein bands shown in Figure 1 were obtained as a result of deconvoluting the ESI spectra of multiply charged ions. The bands shown are the more highly expressed proteins that have been deconvoluted where many of the proteins that are detected at low expression levels have not been fully analyzed or are lost in the noise of the MaxEnt process. Nevertheless, over the full p*I* range, we observed a total of ∼750 unique protein bands between 5 and 75 kDa, with typically 50−60 proteins detected in each p*I* fraction. The bands identified in Figure 1 include many of the common proteins found in cells, such as actin, the tubulin isoforms, Hsp71, Hsp27, vimentin, and NDP kinase, as well as proteins expressed at lower levels. Using this method, we can detect <1 ng of a 40 kDa protein, which is comparable to silver staining methods. In addition, we have obtained each of these p*I* band spectra using an injection of ∼50 ug of protein, which corresponds to $\sim 10^5$ cells. Therefore, we estimate that we can detect proteins that are present at 1000 copies per cell, which is comparable to that of a silver stained gel, although the 2-D gel method can still detect more proteins over a larger MW range. The mass mapping method is highly efficient below 40 kDa, but loses sensitivity at increasing MW as a result of the increased number of multiple charges for larger proteins. Improvements in detection of proteins will be accompanied by enhancements in the deconvolution process for the ESI-multiply charged umbrellas.

Table 2. Purported Proteins Not Identified by MALDI but Present in Fraction 6 in Both ES2 and OSE

OSE MW	% B	ES2 MW	% B
12 646	36.206	12 648	36.239
13 750	36.439		
15 852	36.556	15 856	36.539
8963	37.223	8964	37.089
20 730	37.69	20 733	37.556
23 160	37.857	23 161	37.69
12 770	38.19	12 772	38.007
16 336	38.323	16 338	38.307
14 601	38.19		
14 678	38.19		
13 859	38.507	13 860	38.474
14 637	39.041	14 634	39.041
38 324	39.041		
16 435	39.074	16 435	39.074
		11 882	39.224
		21 150	39.441
16 627	39.508	16 630	39.441
14 638	39.508	14 636	39.524
36 517	39.641	36 518	39.674
		32714	39.758
45 597	39.808	45 605	39.875
22 289	39.808	22 297	39.875
30 443	39.808	30 448	39.875
16 580	39.774	16 581	39.875
11 828	40.225	51 700	40.175
15 863	40.392	15 866	40.558
32 849	40.625	32 851	40.558
9972	40.942	9975	40.992
38 132	41.692	38 130	41.742
31 040	42.409	31 048	42.376
12 173	43.31	12 176	43.61
		36 027	43.793
		30 097	44.027
23 220	44.111	23 221	44.327
		14 207	44.477
27 311	44.776	27 310	44.944
35 700	45.461	35 705	45.428
26 847	45.461	26 847	45.311
29 920	46.195	29 922	46.112
47 987	46.695	47989	46.729
28 694	46.195	55 961	47.579
29 725	47.212	16 522	47.863
57 979	49.681	57 991	49.53
27 281	50.097	27 288	49.814
29 627	50.047	29 629	49.897
36 559	50.181	36 569	50.248

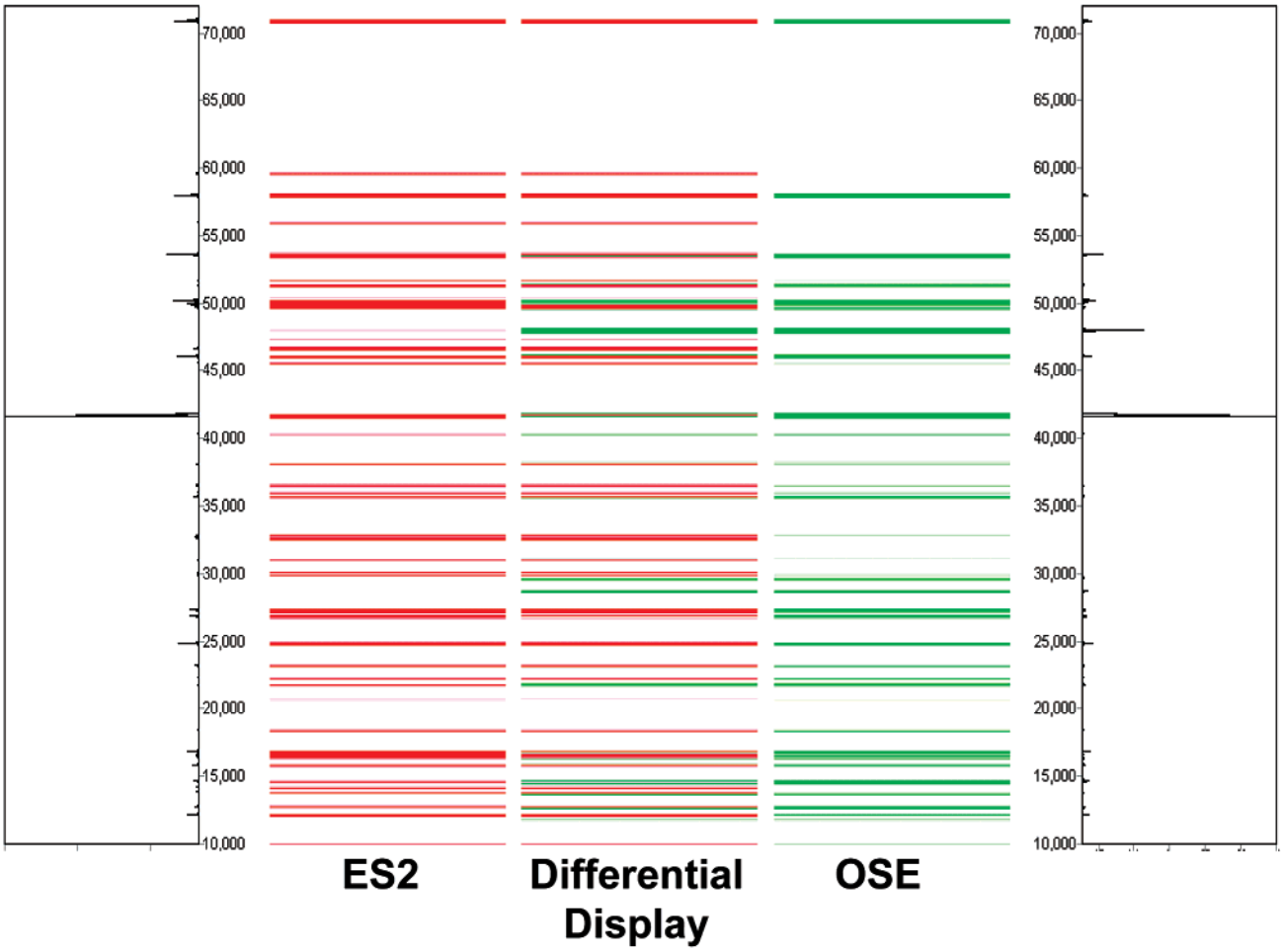

Figure 3. Differential display map between the ES2 cell line and OSE cell line for fraction 6 from the Rotofor. The ES2 cell line is displayed with the protein bands in shades of red, and the OSE cell line is displayed with the protein bands in shades of green. The lane in the center is the differential display where the intensities of the protein bands of the same MW have been subtracted from one another.

The key advantage of the 2-D liquid separation/mass mapping method over 2-D gels is the use of an accurate MW as the standard for interlysate comparison. A major problem with 2-D gels in interlysate comparisons is the lack of run-to-run reproducibility. This problem is accentuated in interlaboratory comparisons where one may want to compare a protein profile image to that of an image in a database. The use of the MW of the intact protein becomes the standard for comparison. This is shown in Figure 1, where proteins have been mapped according to MW, and these proteins can be compared between OSE and an ovarian cancer cell line. In 2-D gel electrophoresis, if the gel spots run differently for each sample, then the spots are generally compared by selecting standard proteins as markers and comparing the protein patterns. However, this process often proves unreliable if the identity of the spots is unknown and there are major changes in spot intensity. The accurate MW generated by our method tags the protein with a highly reproducible marker within 100−200 ppm using modern ESI-TOF mass spectrometers. The MW together with the p*I* and the hydrophobicity can provide a means of tagging a protein for changes in expression in interlysate comparisons.

In these interlysate comparisons, the proteins are compared on the basis of the p*I* fraction and the intact MW of the protein. In addition, there is also the hydrophobicity, which can be compared in terms of percent acetonitrile elution. Thus, there are at least three parameters in which to intially compare the proteins in each fraction. In the mass maps of Figure 1, the proteins have been identified using peptide mapping by MALDI-TOFMS and database searching. The database MW can then be compared to the experimentally determined MW using the ESI-TOFMS. Table 1 lists the proteins that have been identified in both the pooled OSE and cancer cell lysates from Rotofor fractions 6, 7, and 14. The MW of a protein in each fraction is often detected within 100−200 ppm from that of the other fraction. Examples include vimentin, which is detected as MW 53 586 Da in the OSE fraction and 53 580 Da in the ES2 cell line. This is a difference of 6 Da or ∼110 ppm, which suggests that it is the same protein in both samples; a conclusion verified by peptide mapping with nearly 40% coverage. The MS fit MW is given as 53 686 Da. Although the experimental MW is close to the database value, a difference of >100 Da indicates the probable presence of as yet unidentified posttranslational modifications. Further sequencing work will be required to determine the exact nature of the putative posttranslational modification. Another example is Hsp71, which had an experimental MW of 70 890 and 70 891 Da for OSE and ES2, respectively. Remarkably, the difference in Hsp71 MW between OSE and ES2 was only ∼14 ppm. The Msfit database value of the intact MW is 70 898 Da, which is very close to the measured value.

Another protein in fraction 6, cytokeratin 7, has an experimental MW of 51 336 and 51 337 Da for OSE and ES2, respectively, which almost exactly matches the database value of 51 335 Da. The key issue is that for interlysate comparisons, proteins can be matched on the basis of the p*I* and, importantly, an intact and highly accurate MW. The protein matches can then be verified by peptide mapping, which confirms the identity of the protein on the basis of predicted peptide maps generated by virtual digests.

It should be noted that most of the proteins in Table 1 have MWs that differ from the database by between 0.1 and 3 parts/thousand. The database value itself does not affect these comparisons, since we are interested in matching protein MWs between different cell lines. However, it does indicate that many of the proteins observed in human cells may be modified in some manner. Identification of some of these modifications in Hela cells has been discussed in previous work.[25] A comparison between the Hela cell MW profile to that of the ES2 map indicates that a number of the common proteins observed in both of these cancer cell lines have very similar MW values and may be modified in a similar manner. Two important modifications in cancer involve phosphorlylations and changes in protein structure between normal and cancer cells. Using the intact MW generated by NPS RP-HPLC followed by ESI-TOFMS compared to the database value often results in a difference of 80 Da or some multiple of 80 Da, which suggests, among several possibilities, the presence of one or more phosphorylation groups. This can be observed, for example, in fraction 6 for ran-specific GTPASE-activating protein and tubulin β-1-chain, where differences of close to 80 Da are observed within the expected error of the measurement. The actual presence and location of phosphorylation sites must be confirmed by detailed sequencing methods, but the MW values serve as valuable indicators of where in the proteome to look for phosphorylations. In addition, most of the proteins detected in both OSE and ES2 have the same MW within 100−150 ppm accuracy to which we are limited in the present experiment. In the fractions under study, few proteins show significant shifts in MW between OSE and ES2.

The measured intact protein MW values in each fraction are sufficiently close to each other so that for interlysate comparisons, one might expect to be able to directly compare these proteins on a mass map, such as that in Figure 1. The main source of error in the MW value is due to calibration drifts where a lock mass is used only at selected points in the chromatogram. The use of a continuous lockmass during the RP-HPLC/MS run currently available using a second ESI source on newer models of the LCT ESI-TOFMS would improve the run-to-run calibration. However, in each of these interlysate comparisons, the proteins are matched to each other on the basis of a hydrophobicity parameter in addition to the MW and p*I*. This is demonstrated in Figure 2, which shows the NPS RP-HPLC chromatograms for the separations of proteins from Rotofor fraction 7 for OSE versus ES2. In this case, fractions were collected for digestion followed by peptide mapping. On the basis of the percent acetonitrile, which is very reproducible, and the presence of several common marker peaks in the chromatogram, one can readily match the proteins collected. Even proteins with similar MW values often have very different hydrophobicities and are readily distinguished by HPLC. The acetonitrile gradient from the RP NPS HPLC can be aligned with

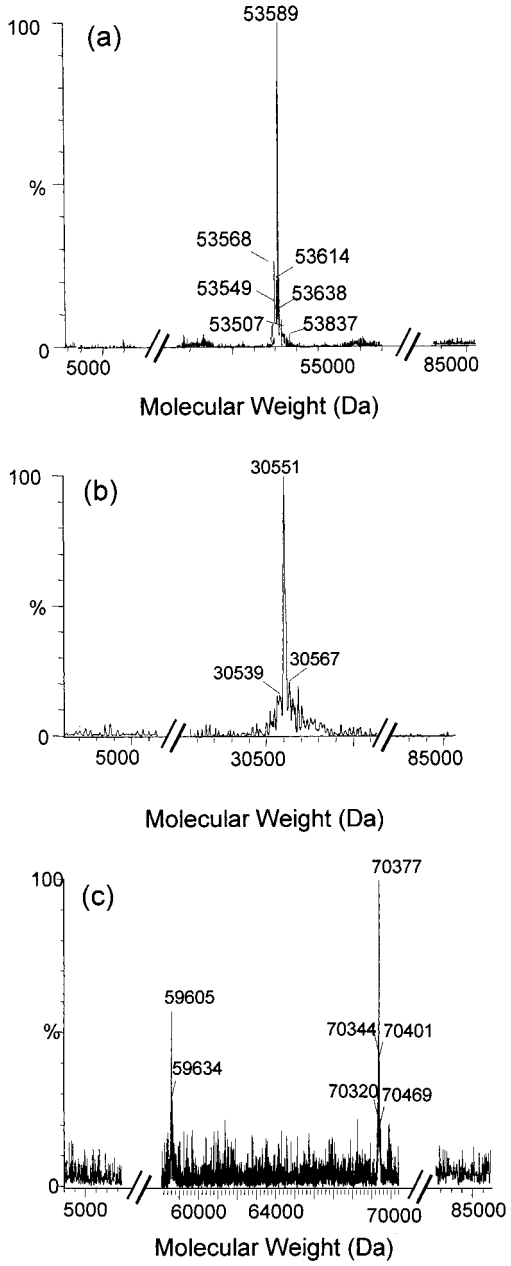

Figure 4. ESI-TOFMS of intact MW of (a) vimentin at 53 589 Da, (b) unidentified protein of MW 30 551 Da, and (c) two proteins that coelute, identified as T-plastin at 70 377 Da and T-complex protein at 59 605.

the on-line LCT separation and detection of proteins so that the MW peaks can be associated with a third parameter, which is percent B, as shown in Figure 2. The protein peaks in the chromatograms that have been identified (by collection, digestion, and peptide mapping) can be readily matched between the two cell lines. The importance of the use of percent B is shown in Figure 1c for a band around 58 kDa, which contains three proteins of similar MW: M1 pyruvate kinase (57 871 Da), ICAM-1 (57 826 Da), and dyskerin (57 667 Da). These proteins can be easily distinguished on the basis of elutions at different percent B, which are 44.6, 52.4, and 43.2, respectively. Although final verification of identity in these comparisons requires peptide mapping or MS/MS mapping, the three-parameter method was found to provide reproducible matches in every case studied

Table 3. MS-Fit Search Results for T-Plastin and T-Complex Protein 1, ϵ Subunit[a]

m/*z* submitted	MH^+ matched	Delta ppm	start	end	peptide sequence	modifications
14/42 Matches (33%); 70436.2 Da; p*I*, 5.52; Acc. P13797; Human; T-plastin; MOWSE Score, 9.37×10^5 [b]						
1069.6450	1069.6257	18.0109	264	272	(K) LSPEELLLR(W)	
1154.6500	1154.5999	43.4309	124	132	(K) YAFVNWINK(A)	
1233.7180	1233.6843	27.3063	234	244	(K) IGLFADIELSR(N)	
1361.7720	1361.6748	71.3626	580	591	(K) HNNAKYAVSMAR(R)	
1363.7870	1363.7738	9.6817	362	373	(K) LNLAFVANLFNK(Y)	
1414.7950	1414.7847	7.2835	298	309	(K) AYFHLLNQIAPK(G)	
1428.7740	1428.7236	35.3101	516	527	(K) ANDDIIVNWVNR(T)	
1441.7960	1441.7327	43.8921	348	361	(R)QFVTPADVVSGNPK(L)	pyroGlu
1446.8020	1446.7626	27.2061	52	63	(R)EIIQKLMLDGDR(N)	1Met-ox
1556.8610	1556.8623	−0.8310	442	455	(K) VNKPPYPKLGANMK(K)	
1721.9230	1721.8631	34.7640	1	15	(−)MATTQISKDELDELK(E)	
1763.9150	1763.8896	14.3921	69	82	(K) ISFDEFVYIFQEVK(S)	
1867.9350	1867.8860	26.2318	318	333	(R) IDINMSGFNETDDLKR(A)	
1997.0290	1997.1435	−57.3505	593	610	(R) IGARVYALPEDLVEVKPK(M)	
8/42 Matches (19%); 59671.6 Da; p*I*, 5.45; Acc. P48643; Human; T-Complex Protein 1, ϵ Subunit (TCP-1-ϵ) (CCT-ϵ); MOWSE Score, 8.4×10^3 [c]						
1622.9130	1622.9165	−2.1286	401	414	(R) SLHDALCVIRNLIR(D)	
1667.9280	1667.9008	16.2795	353	368	(K) LGFAGLVQEISFGTTK(D)	
1738.9770	1738.9492	15.9898	324	340	(R) WVGGPEIELIAIATGGR(I)	
1943.9890	1943.9571	16.4313	184	201	(R)QMAEIAVNAVLTVADMER(R)	pyroGlu
1961.0270	1960.9836	22.1279	184	201	(R) QMAEIAVNAVLTVADMER(R)	
1994.0250	1994.1174	−46.3273	143	160	(R) VAIEHLDKISDSVLVDIK(D)	
2171.1370	2171.1211	7.3293	1	20	(−)ASMGTLAFDEYGRPFLIIK(D)	Acet N
3135.5450	3135.5165	9.0998	450	478	(R) AFADALEVIPMALSENSGMNPIQTMTEVR(A)	

[a] Paramenters used: PI (all); MW 1000−80 000; 75 ppm mass error; max no. missed cleavages, 1; cysteines unmodified; N-terminus, H; C-terminus, OH. [b] The matched peptides cover 28% (180/627AA's) of the protein. [c] The matched peptides cover 24% (132/541AA's) of the protein.

herein. It should be noted that in the present work in interlysate comparisons using 2-D gels, protein spots are compared based upon marker proteins and relative spot positions on the gel image. The three-parameter mapping method provides a much more exact method of comparing proteins from cell lysates. Thus, using our mass mapping technique, a MW map of a cell line can be used as a standard against which other cell lines can be compared.

The use of the three-parameter method becomes especially important in cases in which the proteins cannot be readily identified by peptide mapping methods and database searching. This is shown in Table 2, which lists the MW values of proteins in fraction 6 that have not been identified by peptide mapping. Many of the unknown proteins listed in Table 2 have very similar (within 150 ppm) MWs in both pooled OSE and ES2. Although these proteins share p*I* and very similar MW values, without identities (by MALDI peptide mapping and database searching), we cannot be certain if they are the same protein. Moreover, determining protein identity by MALDI peptide mapping followed by database searching is not without error. The liquid phase separation technique presented here provides a third parameter for matching unknown proteins from different sources.

Proteins can be matched on the basis of their hydrophobicities. As shown in Table 2, some proteins found in OSE and ES2 have identical p*I*s and very similar MWs and hydrophobicities (within <0.1% B). In addition, these proteins can be enzymatically digested, and their peptide maps can be compared to further support or disprove the hypothesis that they are the same protein. If a protein has not been identified by MS techniques, yet the three-parameter method presented in this work suggests that a protein is common to two samples, then the quantitative measure of upregulation or downregulation between two samples will help to determine if the protein is sufficiently important to warrant further investigation. In other cases, there are proteins that appear in one fraction or the other. These are potential marker proteins, and further work will be required to identify these proteins.

A second important issue is the quantitation of proteins in interlysate comparisons. The ESI efficiency is different for each protein; however, in these differential expression studies, we are comparing the same proteins between different samples rather than proteins within the same sample. To normalize the quantitation between different runs, an internal standard, insulin, has been used. In addition, several of the major housekeeping proteins can be compared, since these generally do not change significantly in expression. This method is often used for quantitation methods in 2-D gel patterns. The result is that we can compare protein bands in terms of intensity and MW so that changes in expression and structure can be determined. The relative changes in quantitation are shown in Table 1 in terms of a percent change between the proteins in the OSE versus the ES2 cell line. A 100% change means a factor of 2× in expression. Many of the proteins in fraction 6 show only relatively small changes between 1 and 3× in upregulation, as compared to the normal cells. However, in many other cases, there are strong differences in expression between proteins in the OSE versus ovarian cancer cells. In fraction 7, there are two bands around 69 kDa identified as ezrin (P81) and fragile X mental retardation syndrome related protein 1. Ezrin is detected in the normal cells but in a different fraction when compared to ES2 cells, but the fragile X mental retardation syndrome related protein 1 is detected in the ES2 cells, but not in normal cells. Inorganic phosphatase, acetylcholine receptor protein, β-chain precursor, DNA polymerase ϵ-subunit B and KIAA0513 are expressed in ES2 fraction 6, but not in the

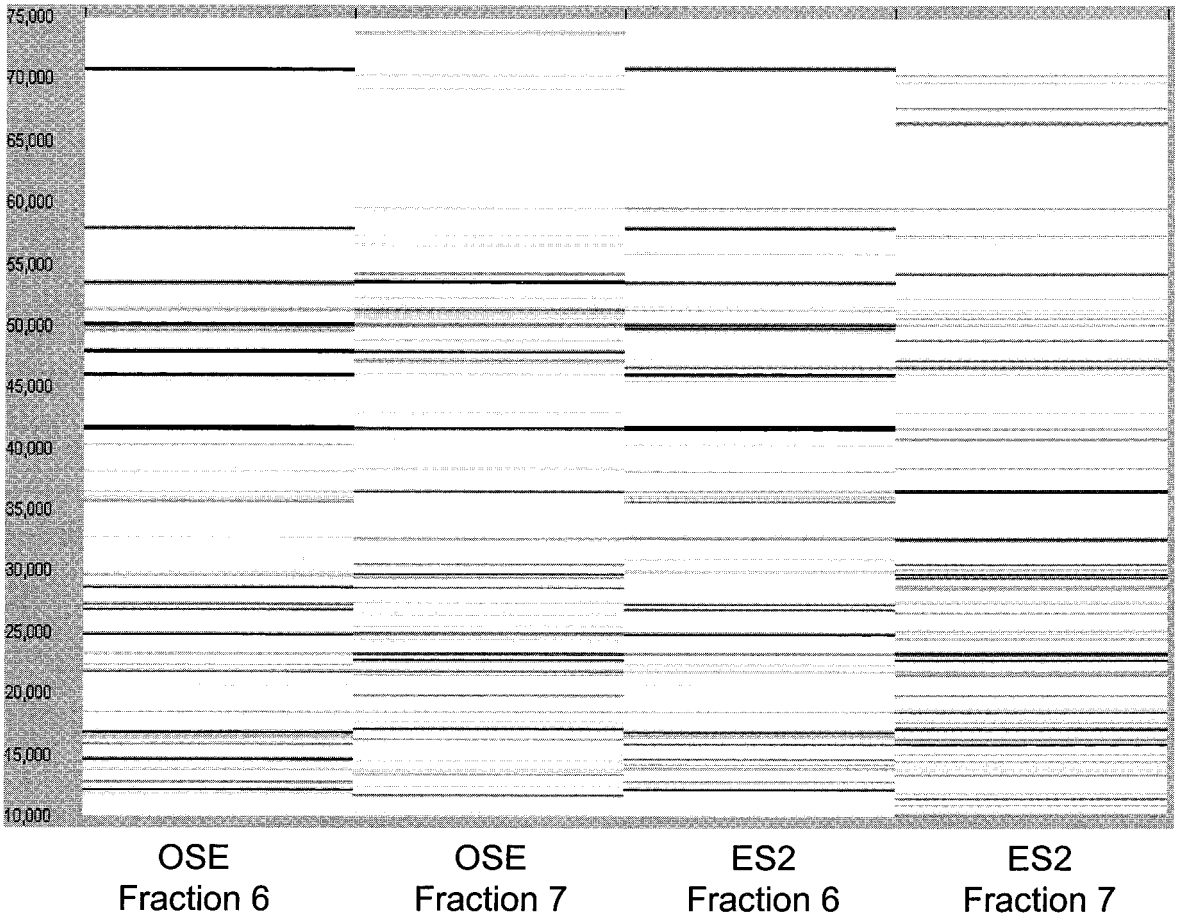

Figure 5. Comparison of the mass maps for fractions 6 and 7 between the OSE cell lines and the ES2 cell lines, demonstrating the limited overlap between the fractions.

corresponding normal sample. KIAA0513 appears very promising as a potential cancer marker in this cell line because of its strong expression in the ES2 cells and not in the normal cells. Other proteins are differentially expressed in varying degrees, as shown in Table 1. Many of these have not yet been identified, such as the two proteins in fraction 7 around 68 kDa that are strongly expressed in the ES2 cell line, but not in the normal fraction. Cancer researchers have generally been limited to searching for differences of at least a factor of 2 from gels. Using the mass-mapping method detection of more subtle differences in protein expression will also be possible. The validation of such markers and establishment of the biological significance of subtle expression differences will clearly require extensive studies on large numbers of such samples. However, the 2-D liquid separation/mass mapping method can provide a reproducible method of comparing the expression of intact proteins from cellular samples on the basis of their MWs. In addition, using internal standards, any number of related cell lines can be compared against a standard mass map. This compares to other methods, such as quantitative differential two-color fluorescence display methods in 2-D gels or the comparative isotope-labeled quantitation methods, such as ICAT, in which only two samples can be compared at the same time.

An important issue is the ability to provide a method for differential display of the 2-D mapping technique so that changes in protein expression can be readily measured. In Figure 3 is shown a 2-D differential map of fraction 6 in which the ES2 cell line has been mapped in shades of green and the normal cell line, in shades of red, where the intensity of the peak is related to the protein expression. The differences between the intensities are shown in the center lane where the shades of red or green show the quantitative differences between protein expression in the cancer versus normal samples. The center lane clearly shows proteins that are highly expressed in the ES2 cell line and not in the normal sample as bright red bands, and proteins that are highly expressed in the normal sample but not in the cancer cells are shown as bright green bands. Proteins that may be present in both samples are shown in less intense shades of green or red. Using this method, we can readily detect differences in protein expression, limited mainly by the reproducibility of the first dimension. The differential display method will allow the pooled OSE cell lines to be used as a future standard against which other ovarian lines can be compared on the basis of the intact MW. In addition, changes in protein structure can often be observed by differences in MW, as reflected by shifts in bands. This, of course, must be corroborated by peptide mapping or MS/MS identification.

An important issue in these experiments is the degree of fractionation that is achieved prior to mass detection. This issue is important in terms of quantitation and identification. In the mass spectrometer, at any point sampled in time, there is generally only one major MW peak detected, as shown in Figure 4. There may

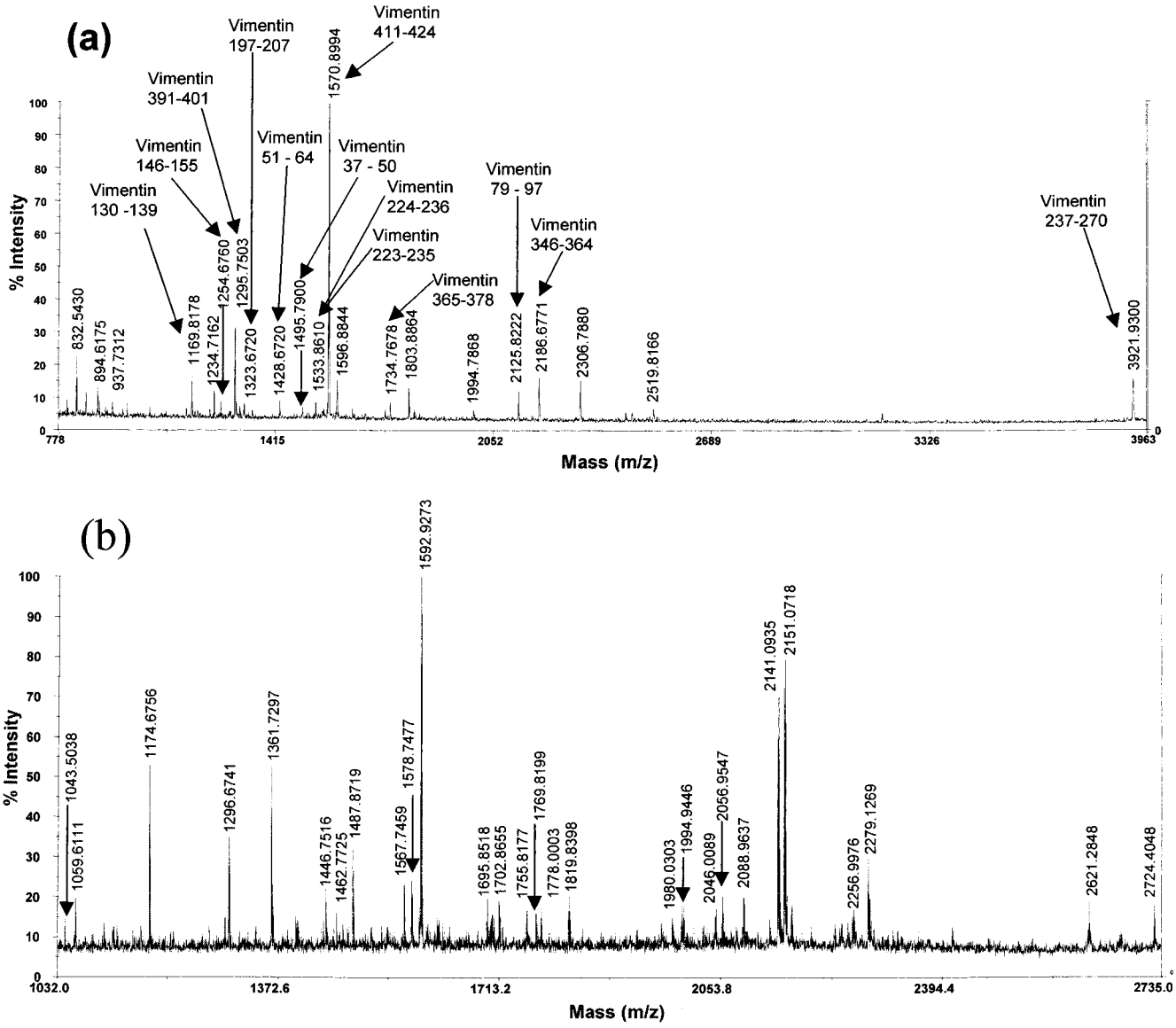

Figure 6. MALDI-TOFMS peptide map of (a) vimentin and (b) unidentified protein at MW 30 551.

also be additional minor peaks detected, although in these figures, only the MW peak region is shown, since there are no other significant peaks present. There is sometimes an additional major peak detected, as shown in Figure 4c. Even though these two proteins have similar hydrophobicities and are not easily separated by HPLC, they are separated by MW in the ESI-TOF. When enzymatic digestion is performed on the coeluted proteins collected in one fraction, the peptides detected by MALDI-MS indicate the presence of more than one protein. This is illustrated in Table 5, the MS-Fit search results for the fraction that included the two coeluting proteins shown in Figure 4c. Table 5 shows that a completely separate set of peptides is matched for T-plastin, as compared to T-complex 1, ϵ subunit, indicating that the trypsin digest contains these two proteins. The MS-Fit results indicate a MW of 70 436 and 59 671 for T-plastin and T-complex protein 1, ϵ subunit, respectively, which is close to the LCT MW for each of these proteins, as shown in Figure 4c. Although there is usually one major peak in each fraction detected by MS, additional prefractionation may be needed for further separation of proteins, especially when trying to detect low-abundance proteins.

An issue in the use of the Rotofor has been incomplete fractionation due to diffusion between the compartments. A comparison of the protein maps from two adjacent pH fractions from the Rotofor is shown in Figure 5. There are some proteins that overlap in both fractions, such as actin, which is a very high abundance protein, in which the main protein band is in fraction 6, but there is a protein band of much lower abundance in fraction 7. However, there are many bands in which there is little or no overlap, such as Hsp 71 in fraction 6 or actin-like protein in fraction 6. In these cases, there is no overlap band detected in the adjacent fraction. The fractionation by the Rotofor appears to be relatively clean in the mid-pH fractions. The overlap between adjacent fractions becomes more serious at high pH because of cathodic drift. This drift is also a problem at high pH in 2-D gel work. The use of a device utilizing compartments separated by membranes of well-defined pH values, such as that used in the commercial Isoprime device,[34,46] would provide improved fractionation in the pH dimension.

(46) Righetti, P. G.; Bossi, A.; Wenisch, E.; Orsini, G. *J. Chromatogr. B* **1997**, *699* (1−2), 105−115.

An unresolved problem in current work is the identification of protein bands that are detected in the ESI-TOFMS as intact MW values but which cannot be identified by MALDI-TOFMS tryptic mapping. In these studies of human cell lines, <40% of the proteins detected could be identified with satisfactory results from the databases. An example of the quality of the MALDI-TOFMS data is shown in Figure 6a,b for two proteins, one of which has been identified as vimentin and one of which remains unidentified at MW 30 551 Da. The MALDI-TOFMS data were obtained using standards such that the mass accuracy for peptides was typically 10−20 ppm. The coverage of the proteins generally varied between 20 and 60%. An important advantage of the liquid separations shown in prior work is that purified proteins from liquid separations need minimal cleanup, as compared to proteins from gels, and generally provide improved coverage and signal-to-noise ratios and fewer artifactual modifications, such as oxidations, that are often found in protein from gels.[23] In addition, the proteins were highly denatured during the HPLC process by the use of denaturing solvents and the use of 60 °C in the separations. The use of liquid separations in denaturing solvents at elevated temperatures has been shown to provide efficient and rapid tryptic digestions relative to that in aqueous solutions, for example.[47] In addition, DTT was added to break disulfide bonds, but in many cases, it provided only limited improvement in coverage. The other possible problem could be that multiple proteins were present in a fraction so that peptides were present for more than one protein. However, as shown in Figure 4a,b, this was not found to be the case in which the MW mass spec for each of the proteins corresponding to the maps in Figure 6a,b are shown to contain one major protein. Further identification work will require MS/MS and searching against other databases.[48] In addition, since the whole protein can be collected as purified in the liquid state, then further sequencing using LC or CE/MS/MS, Edman sequencing, and amino acid analysis can be performed.

CONCLUSION

This method will provide the biomedical research community and cancer biologists with a virtual image of hundreds to perhaps thousands of protein features within a cell lysate and, importantly, a rational approach for making interlysate comparisons on the basis of highly accurate MW determination. In addition, liquid-phase separation has the potential to be automated, thereby allowing efficient and timely comparison of multiple samples. In the postgenomic era, the need to focus on the numerous functional changes to proteins in the cancerous cell is obvious. Hopefully, this method will help identify putative cancer biomarkers that can be used for early detection and prognostication, especially for cancers such as ovarian adenocarcinoma, which is rarely detected early and has an extremely poor five-year survival outcome if detected as advanced disease. The comparison between ES2 ovarian carcinoma cells and a pool of four ovarian surface epithelial cell lines presented here demonstrates the potential of this technique. Numerous differentially expressed proteins were easily visualized, and because liquid-phase separation lends itself to mass spectrometry techniques, many of the proteins were identified with high confidence. The utility of this technique is further supported by the observation that some of the identified and abundantly overexpressed proteins in the ES2 cell line, as compared with normal OSE, had corresponding transcript levels that were unchanged or similar between ES2 and OSE (Cho laboratory, unpublished data). This suggests that important differences between cancer cells and normal cells may be discovered only by profiling the proteome, a task that may be less daunting through the application of the methods presented herein.

(47) Russell, W. K.; Park. Z. Y.; Russell, D. H. *Anal. Chem*. **2001**, *73* (11), 2682−2685.
(48) Yates, J. R. *J. Mass Spectrom*. **1998**, *33*, 1−9.

ACKNOWLEDGMENT

We gratefully thank Dr. Tim Barder of Eichrom Technologies, Inc., for helpful suggestions during the course of this work. We also thank Tim Riley and Matt Dilts of Micromass, Inc. for the loan of the LCT. We also thank Dr. Phillip Andrews of the University of Michigan and Dr. Rick Edmondson of Proteomic Research Services for the use of their MALDI-TOFMS facilities. The authors gratefully acknowledge support of this work in part under the University of Michigan Program in Bioinformatics Pilot Research Grant (D.M.L., K.R.C., D.R.S.), the National Institutes of Health under grant R01GM49500 (D.M.L.), the National Science Foundation under grant DBI-9987220 (D.M.L.), and the National Cancer Institute under grant U19 CA84953 (K.R.C.).

Received for review November 6, 2001. Accepted January 30, 2002.

AC011159C