Evaluation of Two-dimensional Differential Gel Electrophoresis for Proteomic Expression Analysis of a Model Breast Cancer Cell System*

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The technique of fluorescent two-dimensional (2D) difference gel electrophoresis for differential protein expression analysis has been evaluated using a model breast cancer cell system of ErbB-2 overexpression. Labeling of paired cell lysate samples with *N*-hydroxy succinimidyl ester-derivatives of fluorescent Cy3 and Cy5 dyes for separation on the same 2D gel enabled quantitative, sensitive, and reproducible differential expression analysis of the cell lines. SyproRuby staining was shown to be a highly sensitive and 2D difference gel electrophoresis-compatible method for post-electrophoretic visualization of proteins, which could then be picked and identified by matrix-assisted laser-desorption ionization mass spectroscopy. Indeed, from these experiments, we have identified multiple proteins that are likely to be involved in ErbB-2-mediated transformation. A triple dye labeling methodology was used to identify proteins differentially expressed in the cell system over a time course of growth factor stimulation. A Cy2-labeled pool of samples was used as a standard with all Cy3- and Cy5-labeled sample pairs to facilitate cross-gel quantitative analysis. DeCyder (Amersham Biosciences, Inc.) software was used to distinguish clear statistical differences in protein expression over time and between the cell lines. ***Molecular & Cellular Proteomics 1:91–98, 2002.***

The ability to determine statistically significant alterations in protein expression that correlate with disease or occur consequent to experimentally induced changes in cells is fundamental to the exploitation of proteomics. Previous studies from our laboratory have used two-dimensional (2D)[1] gel analysis of immunomagnetic affinity-sorted primary human breast cells to establish the protein expression profiles of luminal and myoepithelial cells (1). These differential expression studies were carried out using replicate 2D gels of each sample and post-staining with a fluorescent protein dye. The subsequent detailed curation and correlation of images was used to derive data sets for statistical analysis. This approach was attractive, because the fluorescent protein stain has a wide linear range of detection, thus giving improved quantification of both high and low abundance proteins. However, in these experiments, many pairs of gels were required to establish statistically significant differences in protein expression as each gel contains inherent experimental variations that limit image superimposition.

The introduction of fluorescent 2D differential gel electrophoresis (DIGE) by Unlu *et al.* (2) has now made it possible to detect and quantitate differences between experimental pairs of samples resolved on the same 2D gel. The basis of the technique is the use of two mass- and charge-matched *N*-hydroxy succinimidyl ester derivatives of the fluorescent cyanine dyes Cy3 and Cy5, which possess distinct excitation and emission spectra. These are used to differentially label lysine residues of two protein samples for comparative analysis of the mixed sample on one gel. The ability to directly compare two samples on the same gel not only avoids the complications of gel-to-gel variation but also enables a more accurate and rapid analysis of differences and reduces the number of gels that need to be run. This procedure has been further developed by Amersham Biosciences, Inc. and has been evaluated recently in an *in vivo* mouse toxicology study (3).

Labeling reactions are carried out under conditions where proteins are "minimally" labeled, such that only 20% of molecules of a particular protein are covalently modified with one Cy dye molecule. Detection of proteins for excision and mass spectroscopy requires post-staining of gels with a general protein stain, because the unlabeled majority of a protein will not exactly co-migrate with the labeled protein, particularly in the low molecular weight range. A third fluorescent Cy dye (Cy2) has also been introduced, making it possible to com-

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[1] The abbreviations used are: 2D, two-dimensional; DIGE, differential gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; MS, mass spectroscopy; HRGβ1, heregulin β1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; AmBic, ammonium bicarbonate; Hsp, heat shock protein.

pare three samples on one gel. An experimental design that should allow a much more accurate statistical analysis of expression differences across multiple gels can now be developed, because different Cy3- and Cy5-labeled samples can be compared with a Cy2-labeled "standard" run on every gel.

In this paper we describe further evaluation of the 2D-DIGE technique and seek to extend our protein expression studies in breast cancer. Specifically we investigated ErbB-2-mediated transformation in a model cell line system comprised of an immortalized luminal epithelial cell line and a derivative stably overexpressing ErbB-2 at a similar level to that seen in breast carcinomas (4). The ErbB-2 receptor tyrosine kinase (also known as neu/HER2) is overexpressed in 25–30% of breast cancers and is often associated with poor prognosis. Differentially expressed proteins detected in this cell system are likely to be involved in the processes of ErbB-2-mediated transformation. In the first experiments we evaluated the feasibility of the technique for monitoring protein expression changes in a model cell line system and tested its utility for high sensitivity, high throughput differential expression proteomics. We have examined the sensitivity and reproducibility of Cy3/Cy5 dye labeling and employed SyproRuby (Molecular Probes) gel staining to visualize proteins for automated spot picking. We have identified a number of differentially expressed proteins resulting from ErbB-2 overexpression using matrix-assisted laser-desorption ionization (MALDI) mass spectroscopy (MS). In a second set of experiments, we introduced Cy2 dye labeling of a standard pooled sample for linking gel images of pairs of samples from differentially treated cells. In this case we have employed proprietary software from Amersham Biosciences, Inc. (DeCyder) to collect, process, and derive statistical data in an experiment that examines the effect of a growth factor on the expression profile of a normal, mammary luminal epithelial cell line *versus* its ErbB-2-overexpressing derivative.

EXPERIMENTAL PROCEDURES

Cell Culture and Mitogen Stimulation—The parental HB4a cell line was established from flow-sorted normal human breast luminal epithelial cells by immortalization with a non-DNA binding, temperature-sensitive mutant of SV40 large T-antigen (U19tsA58) (5). The ErbB-2-overexpressing variant HBc3.6 clone was derived from HB4a by stable co-transfection with a full-length normal human ErbB-2 cDNA under control of the murine mammary tumor virus long terminal repeat promoter and an SV40 polyadenylation signal. The HBc3.6 clone was selected, because it overexpresses a level of ErbB-2 similar to that seen in many breast carcinomas of luminal cell origin (4).

Cells were routinely maintained in RPMI 1640 with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen) and 5 μg/ml hydrocortisone and 5 μg/ml insulin (both from Sigma) at 37 °C in a 10% CO_2 humidified incubator. Cells were starved for 48 h in insulin-free medium containing 0.1% fetal calf serum prior to harvesting. Starved cells were also stimulated with 1 nM (8 ng/ml) HRGβ1 (R & D Systems) for 4, 8, and 24 h. The concentration of HRGβ1 used was determined to be the minimum required to induce maximal extracellular signal-regulated kinase 1/2 and Akt phosphorylation after 10 min of stimulation as determined by immunoblotting (data not shown).

Sample Preparation and Protein Labeling—Cells at ~80% confluence were washed twice in 0.5× phosphate-buffered saline, lysed in lysis buffer (4% (w/v) CHAPS, 2 M thiourea, 8 M urea, 10 mM Tris-HCl, pH 8.5), and then homogenized by passing through a 25-gauge needle six times. Insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 10 °C. Protein concentration was determined using the Coomassie protein assay reagent (Pierce).

Cell lysates were labeled with *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3, and Cy5 (Amersham Biosciences, Inc.) following the protocol described previously (3). Typically, 100 μg of lysate was minimally labeled with 400 pmol of either Cy3 or Cy5 for comparison on the same 2D gel. Labeling reactions were performed on ice in the dark for 30 min and then quenched with a 50-fold molar excess of free lysine to dye for 10 min on ice. Differentially labeled samples were mixed and reduced with 65 mM dithiothreitol for 15 min. Ampholines/pharmalytes, pH 3–10 (1% (v/v) each; Amersham Biosciences, Inc.), and bromphenol blue were added, and the final volume was adjusted to 350 μl with lysis buffer. For the HRGβ1 stimulation experiments, lysates from three separate time course experiments were run in parallel. The triplicate sets of cells were serum-starved for 48 h and then stimulated for 4, 8, and 24 h with 1 nM HRGβ1 or left unstimulated (0 h). The 24 lysates generated were labeled with Cy3 (for HB4a) and Cy5 (for HBc3.6). A pool of all samples was also prepared and labeled with Cy2 to be used as a standard on all gels to aid image matching and cross-gel statistical analysis. The Cy3 and Cy5 labeling reactions (100 μg of each) from each time point were mixed and run on the same gels with an equal amount (100 μg) of Cy2-labeled standard. Thus, the triplicate samples and the standard were run on 12 gels (*i.e.* three gels with two cell lines from each of the four time points), to generate 36 images.

Protein Separation by 2D Gel Electrophoresis and Gel Imaging—Immobilized non-linear pH gradient (IPG) strips, pH 3–10 (Amersham Biosciences, Inc.), were rehydrated with Cy-labeled samples in the dark at room temperature overnight, according to the manufacturers guidelines. Isoelectric focusing was performed using a Multiphor II apparatus (Amersham Biosciences, Inc.) for a total of 80 kV-h at 20 °C, 10 mA. Strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS containing 65 mM dithiothreitol and then for 15 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 18 × 20-cm 9–16% gradient or 12% uniform polyacrylamide gels poured between low fluorescence glass plates. Gels were bonded to the inner plate using bind-saline solution (PlusOne) according to the manufacturer's protocol. Strips were overlaid with 0.5% (w/v) low melting point agarose in running buffer containing bromphenol blue. Gels were run in Protean II gel tanks (Bio-Rad) at 30 mA per gel at 10 °C until the dye front had run off the bottom of the gels.

2D gels were scanned directly between glass plates using a 2920 2DMaster Imager (Amersham Biosciences, Inc.). This charge-coupled device-based instrument possesses two six-position filter wheels (excitation and emission) enabling scanning at the different wavelengths specific for each of the Cy dyes and for SyproRuby fluorescent protein stain. An image is built up and converted to gray scale pixel values. Gel images were normalized by adjusting the exposure times according to the average pixel values observed. The images generated were exported as tagged image format (.tif) files for further protein profile analysis.

Post-staining, Image Analysis, and Spot Picking—Gels were fixed in 30% (v/v) methanol, 7.5% (v/v) acetic acid overnight and washed in water, and total protein was detected by post-staining with SyproRuby dye (Molecular Probes) for 3 h at room temperature. Excess dye

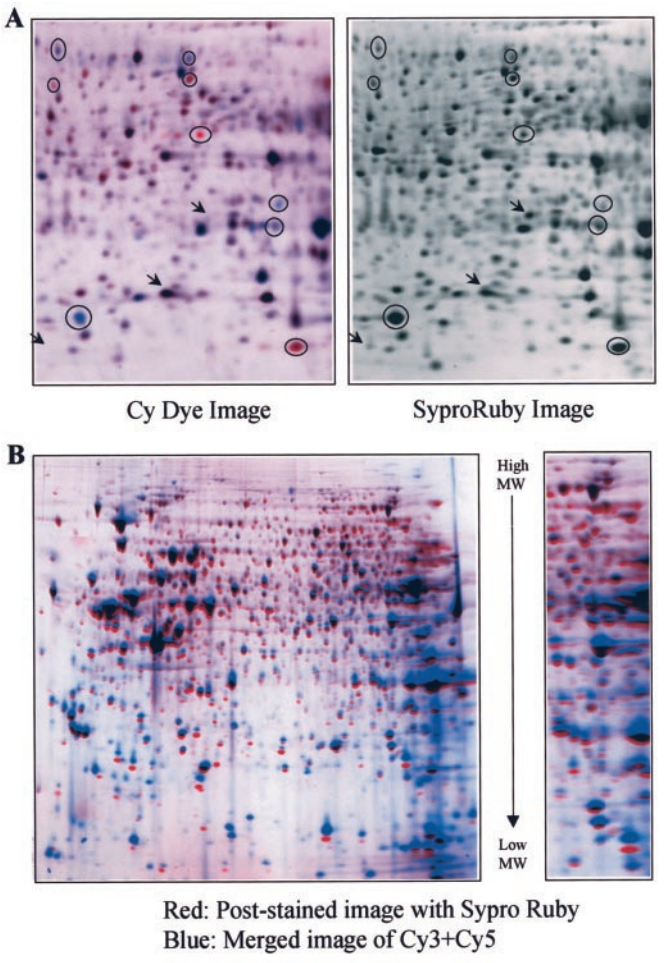

FIG. 1. **Sensitivity of 2D-DIGE and compatibility with SYPRO gel staining.** *A*, comparison of 2D-DIGE imaging and SyproRuby post-staining. Merged Cy dye image of HB4a lysate labeled with Cy3 (*red*) and HBc3.6 lysate labeled with Cy5 (*blue*) (*left panel*). The same gel was post-stained with SyproRuby dye (*right panel*). 100 μg of each lysate from serum-starved cells were analyzed on a 9–16% gradient gel. *Circles* represent differentially expressed proteins detectable by both methods. *Arrows* represent spots detected by SyproRuby but not Cy dye labeling. *B*, the shift in molecular weight between the modified and unmodified proteins was visualized by image overlaying. The DIGE image (*Blue*) was overlaid with the SYPRO image (*Red*).

was removed by washing twice in water, and gels were imaged using the 2920 2D Imager at the appropriate excitation and emission wavelengths for the stain. Gels were also post-stained superficially with silver according to the protocol of Shevchenko *et al.* (6). Images were curated and analyzed using Melanie III (Swiss Institute of Bioinformatics, Geneva, Switzerland) or with DeCyder software (on trial from Amersham Biosciences, Inc.). Differences were also detected visually by direct overlay of images using Adobe PhotoShop (Adobe Systems Incorporated).

For DeCyder image analysis, the differential in-gel analysis mode of DeCyder was first used to merge the Cy2, Cy3, and Cy5 images for each gel and to detect spot boundaries for the calculation of normalized spot volumes/protein abundance. At this stage, features resulting from non-protein sources (*e.g.* dust particles and scratches) were filtered out. The analysis was used to rapidly calculate abundance differences between samples run on the same gel. The biological

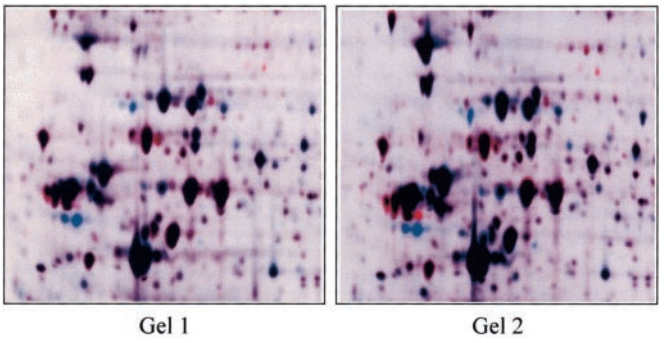

FIG. 2. **2D-DIGE is a reproducible detection method.** Duplicate samples of HB4a and HBc3.6 were labeled separately with Cy3 (*red*) and Cy5 (*blue*), respectively, and run on two separate gels. Images were generated using Adobe PhotoShop (Adobe Systems Incorporated).

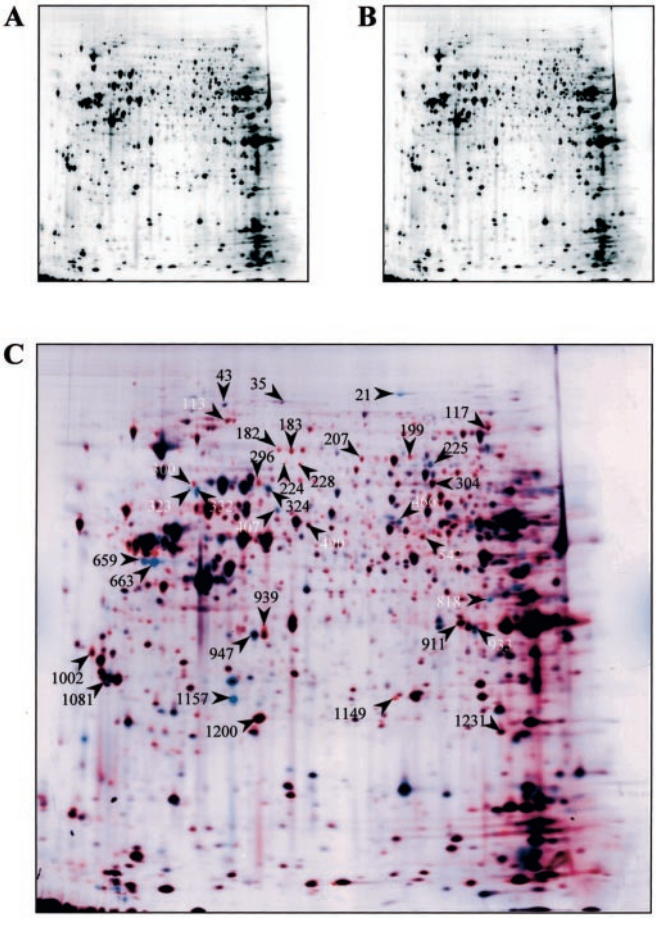

FIG. 3. **Cy dye images of protein expression differences between serum-starved cells.** *A*, HB4a image. *B*, HBc3.6 image. *C*, merged image of HB4a (*red*) and HBc3.6 (*blue*) lysates showing differentially expressed protein spots picked for identification by MALDI-MS and peptide mass fingerprinting. Not all indicated proteins have been identified (see Table I). The image shown is of a 200-μg sample (100 μg each of Cy3- and Cy5-labeled lysates) run on a pH 3–10 non-linear gradient IPG strip and 9–16% gradient polyacrylamide gel.

TABLE I

Summary of differentially expressed proteins in ErdB-2-overexpressing cells identified by MALDI-MS

The table shows identified proteins that were significantly up- or down-regulated in response to ErbB-2 overexpression. -Fold differences were calculated within the BVA mode of DeCyder using the average standardized abundance values from triplicate spots that had normalized spot volumes of >100,000. Zero time point values (*i.e.* no HRGβ1 treatment) were used for calculations except † and ‡, which were calculated from the 4- and 8-h time points, respectively. All differences are statistically relevant having *p* values of <0.05.

Average -fold difference (HBc3.6 *vs.* HB4a)[a]	Spot no. (Fig. 2*C*)[b]	Protein name	NCBI gene identifier	Predicted M_r	Predicted pI	% Coverage[c]	Match[d]
+18.6	1157	Hsp27	123571	22327	7.83	48	11/36
+13.9	21	Carbamoyl phosphate synthetase	13636759	131192	5.79	38	42/47
+8.5	663	Cytokeratin 17	4557701	48105	4.97	63	31/51
+3.9	332	L-plastin	11434009	70288	5.29	47	25/38
+3.3	466	Glutaminase C	5690372	65474	8.09	45	23/40
+2.0	407	Copine III	4503015	60131	5.60	40	22/63
+1.9	933	Aldose reductase	493797	35706	6.55	32	17/30
+1.4†	35	Leucine-rich protein	1730078	145202	5.50	37	42/72
+1.4	324	T-plastin	2506254	70436	5.52	48	27/68
+1.2	1081	14–3-3b	4507949	28082	4.76	85	32/64
−1.4	542	DNA helicase p50 (RuvB-like 1)	4506753	50228	6.02	45	17/32
−1.4	296	Hsp70	4204880	69995	5.56	49	36/72
−1.4	911	CLP-36	13994151	36071	6.56	65	18/56
−1.5	300	Lamin B	5031877	66408	5.11	58	36/54
−1.5	224	MxA (interferon-induced viral resistance	127566	75534	5.60	66	37/45
−1.6	182	Ku p70/80 subunit (acidic)	10863945	82705	5.55	54	43/63
−1.6‡	1231	Mitochondrial superoxide dismutase 2	11418405	24750	8.34	68	13/71
−1.7	183	Ku p70/80 subunit	10863945	82705	5.55	28	40/93

[a] Average -fold increase (+) or decrease (−) in expression in HBc3.6 *vs.* HB4a.

[b] Spot numbers refer to those in Fig. 2*C*.

[c] Amino acid sequence coverage for the identified protein.

[d] Number of peptide masses matching the top hit from MS-Fit peptide mass mapping *vs.* the total number of masses submitted.

variation analysis mode of DeCyder was then used to match all pairwise image comparisons from difference in-gel analysis for a comparative cross-gel statistical analysis. Operator intervention was required at this point to set landmarks on gels for more accurate cross-gel image superimposition. Comparison of normalized Cy3 and Cy5 spot volumes with the corresponding Cy2 standard spot volumes within each gel gave a standardized abundance. This value was compared across all gels for each matched spot, and a statistical analysis was performed using the triplicate values from each experimental condition.

Changes observed by 2D-DIGE analyses were aligned with Sypro-Ruby protein patterns, and spots were selected for picking according to this post-stained image. Spots of interest were excised from 2D gels using a Syprot automated spot picker (Amersham Biosciences, Inc.) following the manufacturer's instructions. Spots were collected in 200 μl of water in 96-well plates and kept frozen at −20 °C for protein identification by MALDI-MS.

Protein Identification by MALDI-MS—Gel pieces were washed twice in 25 mM ammonium bicarbonate (AmBic) in 50% acetonitrile and dried in a SpeedVac for 10 min. Samples were reduced in 10 mM dithiothreitol, 25 mM AmBic for 45 min at 50 °C and then alkylated in 50 mM iodoacetic acid, 25 mM AmBic for 1 h at room temperature in the dark. Gel pieces were then washed twice in 25 mM AmBic, 50% acetonitrile and vacuum-dried. Proteins were proteolysed with 30 ng of modified trypsin (Promega, Southampton, United Kingdom) in 25 mM AmBic for 16 h or overnight at 37 °C. Supernatant was collected, and peptides were further extracted in 5% trifluoroacetic acid, 50% acetonitrile. Peptide extracts were vacuum-dried and resuspended in 3 μl of water. Digests (0.5 μl) were spotted onto a MALDI target in 1 μl of matrix (2,5-dihydroxybenzoic acid). MALDI-MS was performed using a Reflex III reflector time-of-flight mass spectrometer (Bruker Daltonik, Bremen, Germany) in the reflector mode with delayed extraction. All mass spectra were internally calibrated with trypsin autolysis peaks. Peptide mass mapping was carried out using the MS-Fit program (Protein Prospector; University of California, San Francisco, CA).

RESULTS AND DISCUSSION

2D-DIGE Is a Sensitive and Reproducible Technique for Differential Expression Analysis—Initially, we sought to compare the sensitivity of protein detection using the *N*-hydroxy succinimidyl ester derivatives of the Cy3 and Cy5 dyes with post-staining methods that are compatible with subsequent MALDI-MS identification. In preliminary studies, varying amounts of a standard protein were labeled with Cy dyes, and the sensitivity of detection was compared with that of MS-compatible, superficial silver staining (6). This analysis revealed that Cy dye labeling was in fact more sensitive than silver staining; we could detect less than 1 ng of standard protein by Cy dye labeling compared with 5 ng with the silver staining protocol (data not shown).

Our planned high-throughput approach required bonding of gels to glass plates for automated spot picking. This was to prevent gel shrinkage upon fixation and movement of the gel during picking but also because fluorescent markers used for coordinating the picking process are mounted on the plate. We found that superficial silver staining was even less sensitive on the bonded gels and staining varied considerably from

gel to gel, thus making it unsuitable for 2D-DIGE post-staining. The fluorescent protein stain SyproRuby was much more sensitive than silver, consistently gave uniform staining from gel to gel, and its ability to detect proteins was unaffected by the process of bonding gels to glass plates. SyproRuby was also slightly more sensitive than Cy3 and Cy5 labeling, because a number of proteins detectable by SyproRuby were not easily visualized in the Cy3/5 images (Fig. 1*A*). Although this effect may be because of the enhanced sensitivity of SyproRuby staining, we cannot rule out the possibility that some proteins may not be modified by the Cy dyes as efficiently as others. Using the Melanie III software (7), we detected an average of 1.4 ± 0.1 times more gel features using SyproRuby *versus* Cy dye labeling. Thus, in practice, SyproRuby appears to be an ideal post-stain that is compatible with 2D-DIGE labeling.

Comparison of SyproRuby-stained images with Cy3 or Cy5 images from the same gel revealed that many spots did not exactly align. This was particularly noticeable in the lower molecular weight range of the gels (Fig. 1*B*). This is because proteins are minimally labeled, and the labeled protein has the additional mass of a covalently attached dye molecule (~580 Da). However, this shift was not uniform for all proteins, suggesting that dye labeling could alter the migration or that more than one lysine residue is labeled on that particular protein. For this reason, post-electrophoretic staining was required, and spots of interest were visually aligned prior to picking.

The preliminary Cy dye labeling experiments revealed obvious differences in protein expression between the parental and ErbB-2-overexpressing cell lines, with most of these proteins detectable by SyproRuby post-staining (Fig. 1*A*). For most of the spots of interest, we found that the volume of the spots from Cy dye images correlated with the amount of protein and hence the ability to identify them by MALDI-MS. Several low volume spots that were barely detectable by SyproRuby staining could not be identified by MALDI-MS, and it will be necessary to pick these spots from preparative gels on which more protein is loaded.

We next tested the reproducibility of Cy dye labeling. When the same sample (either an HB4a or an HBc3.6 urea-solubilized lysate) was labeled with Cy3 and Cy5, and the mixed labeled samples were run on the same gels, we detected only very minor differences in the abundance of some proteins (data not shown). These differences were more apparent for the very low abundance proteins. In a further experiment, replicate samples of HB4a and HBc3.6 lysates were labeled with Cy3 and Cy5, respectively, and run on different gels. We were able to detect the same differences in the expression of particular proteins from gel to gel (Fig. 2). These differences were also detectable when the sample-dye combinations were reversed (data not shown). Taken together, these results are in agreement with the conclusions drawn from the more rigorous validation of the technique carried out by Tonge *et al*. (3) and show that this technique is both sensitive and reproducible and can be used for the rapid identification of differences in the protein content of two separate cell lysate samples.

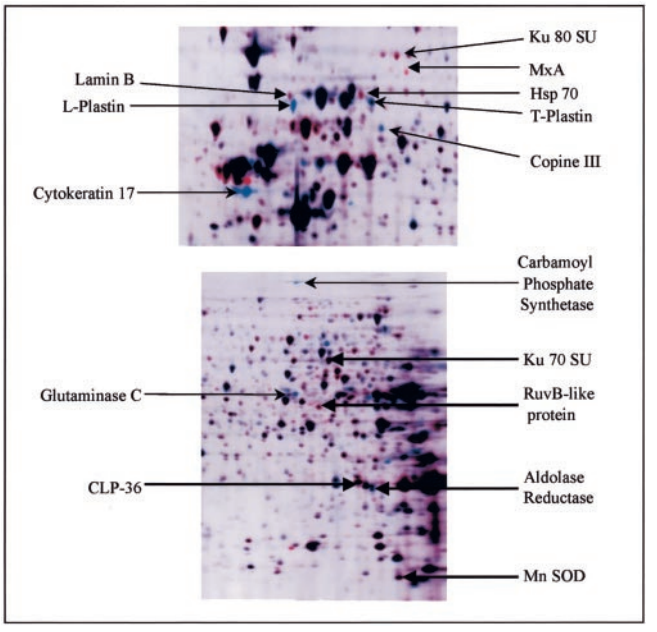

FIG. 4. **2D gel migration patterns of differentially expressed protein identified by MALDI-MS and peptide mass fingerprinting.** Enlarged regions of merged Cy dye images from samples run on pH 3–10 non-linear gradient IPG strips and 9–16% gradient polyacrylamide gels are shown. Identified proteins up-regulated in the HBc3.6 cell line are shown in *blue*, whereas those down-regulated in the HBc3.6 cells are shown in *red*. Refer to Table I for -fold changes in expression calculated using DeCyder software.

Identification of ErbB-2-mediated Protein Expression Changes in Serum-starved Mammary Luminal Epithelial Cells—To identify differences in protein expression resulting from ErbB-2 overexpression, we ran four sets of duplicate gels of HB4a and HBc3.6 lysates labeled with Cy3 and Cy5 in both combinations. We initially chose to compare the expression profiles of only the serum-starved cell pair to simplify experiments and to avoid the problem of sample contamination with bovine serum proteins. Under these conditions, the two cell lines show differences in morphology and proliferative capacity (4), suggesting differential protein expression occurs in the absence of serum. The protein image sets were curated and analyzed using Melanie III. Many of the differences were also detected visually by directly overlaying the Cy3 and Cy5 images using Adobe PhotoShop.

We detected 35 distinct protein spots that showed consistent differences in expression levels between the two cell lines, which were present in all gel images and were detectable by SyproRuby post-staining (Fig. 3). These spots were selected for automated spot picking, and 18 of them were identified with confidence by MALDI-MS and peptide mass fingerprinting (Table I). The migration patterns and differential labeling of the identified proteins are shown on the Cy dye images in Fig. 4. DeCyder software was used to calculate the average -fold

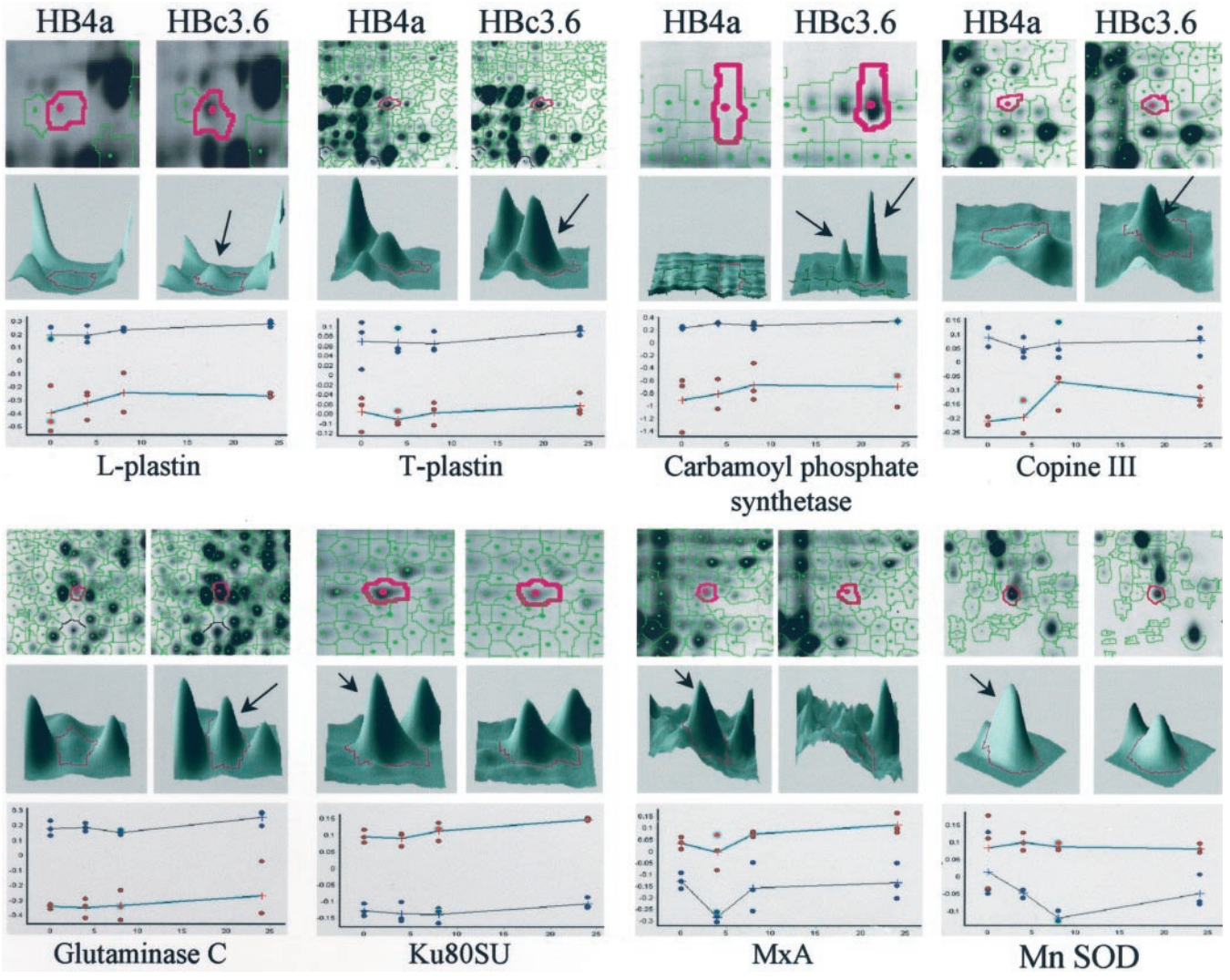

FIG. 5. **DeCyder output showing several of the identified, differentially expressed proteins.** Enlarged regions of images of Cy3-labeled HB4a (*left panels*) and Cy5-labeled HBc3.6 (*right panels*) lysates are shown, with the DeCyder-matched spots of interest highlighted. The three-dimensional fluorescence intensity profiles of the individual spots are shown in the *middle panels*. Graphical representations of all matched spots for a particular protein are shown in the *lower panels*, with changes in expression over time. Values are the standardized log of abundance (*i.e.* log abundance of Cy3- or Cy5-labeled spot over log abundance of Cy2-labeled standard spot). The three points at each time point on the graph represent single values from one gel, and lines are plotted using the averaged values.

difference in expression under serum-starved conditions between the two cell lines (see below). Six of these proteins showed a 2-fold or greater increase in expression in the ErbB-2-overexpressing cell line. Hsp27 showed the greatest increase in expression (Table I). This molecular chaperone protein is involved in various cellular stress responses, apoptosis and actin reorganization, but more significantly, it has been shown to be overexpressed in numerous human cancers, including invasive ductal carcinoma of the breast (reviewed in Ref. 8). Interestingly, Hsp70, another member of the heat shock protein family dysregulated in cancer (8), was down-regulated in the ErbB-2-overexpressing cell line.

Several of the other identified proteins have also been implicated in cellular transformation and metastasis. These are the actin-bundling protein L-plastin (9, 10), the glutamine-metabolizing enzyme glutaminase C (11), and the cell signaling modulator protein 14–3-3β (12). Thus, the up-regulation of these proteins appears to be involved in ErbB-2-mediated hyperproliferation and transformation of this cell type and perhaps confirms that this cell system is a valid cell culture model of breast cancer. We also speculate that increased expression of the metabolic enzymes carbamoyl-phosphate synthetase, glutaminase, and aldose reductase in the HBc3.6 cells is a direct consequence of their enhanced proliferation caused by ErbB-2 overexpression. Moreover, changes in the expression of the identified structural proteins (cytokeratin 17, L-plastin, T-plastin, leucine-rich protein, and lamin B) could account, at least in part, for the different morphologies dis-

TABLE II
Number of protein spots with a significant difference in abundance between cell lines

The total number of matched protein spots with a greater than 1.3-, 1.5-, 2.0-, and 5.0-fold difference in abundance between the cell lines is shown for each time point of HRGβ1 stimulation. The number of spots increased in the HB4a and HBc3.6 cell lines is also shown. Numbers were obtained by filtering data from 2607 matched protein spots within the BVA mode of DeCyder. -Fold differences were calculated from the average standardized abundance of triplicate spots with normalized spot volumes of >100,000. Only averages with *p* values of <0.05 were included.

Time	-Fold difference	Total No. spots	Increased in HB4a	Increased in HBc3.6
h				
T0	>1.3	135	34	101
	>1.5	81	14	67
	>2.0	23	3	20
	>5.0	2	0	2
T4	>1.3	160	53	107
	>1.5	74	22	52
	>2.0	16	2	14
	>5.0	2	0	2
T8	>1.3	159	56	103
	>1.5	68	24	44
	>2.0	20	3	17
	>5.0	3	0	3
T24	>1.3	165	44	121
	>1.5	75	18	57
	>2.0	16	3	13
	>5.0	1	0	1

played by the two cell types. The 18 identified spots were all cut from gels loaded with only analytical amounts (200 μg) of Cy dye-labeled samples that were post-stained with Sypro-Ruby. This suggests that Cy dye labeling and SyproRuby post-staining *per se* do not affect the ability to identify proteins by MALDI-MS. Because mostly unlabeled protein is picked for MS identification using this minimal labeling methodology, it is unlikely that covalent Cy dye modification of lysine residues affects the generation of tryptic peptides. The remaining 17 unidentified proteins are either currently awaiting identification by MALDI-MS peptide mass fingerprinting, or their identities (six of them) cannot be given with statistical confidence because of the low abundance of recovered tryptic peptides. Identification of the low abundance proteins will require resolution of preparative quantities of samples. Ultimately, tandem MS will be applied for identification of differentially expressed protein spots that cannot be identified by MALDI-MS peptide mass fingerprinting.

Effects of HRGβ1 Treatment and ErbB-2 Overexpression on Protein Expression in Mammary Luminal Epithelial Cells: Statistical Analysis of Differential Expression—We further extended our studies to examine the effects of HRGβ1 treatment over time on the expression profiles of the parental and ErbB-2-overexpressing cells (see "Experimental Procedures"). We have recently observed differences in the responsiveness of these cells to HRGβ1,[2] which is a ligand of ErbB-3, the preferred heterodimerization partner of activated ErbB-2 (reviewed in Ref. 13).

TABLE III
Number of protein spots with a significant change in abundance over time

The number of matched protein spots down- and up-regulated in the HB4a and HBc3.6 cell lines over time following HRGβ1 stimulation are shown. Values were obtained by filtering data from 2607 matched protein spots. -Fold differences between each time point and time zero were calculated for each cell line from the average standardized abundance of triplicate spots with normalized spot volumes of >100,000. Only averages with *p* values of <0.05 were included.

Time	-Fold difference	HB4a	HBc3.6
h		*down/up*	
T0/T4	>1.3	5/0	6/1
	>1.5	2/0	0/0
	>2.0	1/0	0/0
T0/T8	>1.3	0/1	2/3
	>1.5	0/0	1/0
	>2.0	0/0	0/0
T0/T24	>1.3	3/6	2/11
	>1.5	3/4	2/4
	>2.0	0/0	0/1

DeCyder analysis showed trends of protein expression in response to HRGβ1 treatment, as well as differences in expression between the two cell lines at each time point (Fig. 5). All of the proteins previously identified by MALDI-MS were also detected by DeCyder software analysis (Table I). The data were filtered to reveal statistically relevant -fold changes in protein abundance (*i.e. p* values of <0.05). There were 135 protein spots with a greater than 1.3-fold average difference in abundance between the two cell lines at the zero time point (Table II). Of these 135 proteins, 34 were higher in the parental cell line (HB4a), whereas 101 were higher in the ErbB-2-overexpressing cell line (HBc3.6). At a 2-fold difference cutoff, there were 23 differentially regulated proteins. The number of significant differences between the cell lines was increased following HRGβ1 stimulation. Overall, the HBc3.6 cell line had a greater number of proteins with increased expression, perhaps correlating with its increased proliferation in response to HRGβ1 treatment. A comparison of the change in expression over time indicated that there were more proteins down-regulated than up-regulated at 4 h, whereas a higher number of proteins were up-regulated than down-regulated at 24 h in both cell lines (Table III).

The abundance of most of the identified proteins (Table I) did not change substantially with HRGβ1 treatment; however notable exceptions were copine III, interferon-induced viral

[2] John F. Timms, Sarah L. White, Michael J. O'Hare, and Michael D. Waterfield, submitted for publication.

resistance protein MxA, and mitochondrial superoxide dismutase 2 (Fig. 5). copine III expression increased in HB4a cells in response to growth factor, whereas its elevated expression remained unchanged in HBc3.6 cells. copine III is a phosphoprotein that possesses an associated kinase activity and exhibits Ca^{2+}-dependent phospholipid binding (14, 15), although its cellular function is unknown. MxA and mitochondrial superoxide dismutase 2 were repressed in the HBc3.6 cells, and their expression was further reduced by HRGβ1 treatment (Fig. 5), suggesting that ErbB-2 signaling negatively regulates their expression. It is imperative to follow up on our findings, using both biochemical and biological approaches to further assess the role of the identified proteins in ErbB-2-mediated transformation. We are also currently trying to identify the additional proteins whose expression is significantly altered by HRGβ1 treatment and ErbB-2 overexpression, as revealed by DeCyder analysis.

In conclusion, we show that 2D-DIGE and DeCyder image analysis is a sensitive, MS-compatible, and reproducible technique for identifying statistically significant differences in the protein expression profiles of multiple samples. This approach was more rapid than conventional analyses that compare multiple, post-electrophoretic stained gels and thus require more runs for statistical certainty. Using this methodology we have identified numerous proteins that are now implicated in ErbB-2-mediated transformation and may represent future targets for breast cancer therapies.

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This manuscript is dedicated to the memory of Craig A. Brooks, who is greatly missed.

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REFERENCES

1. Page, M. J., Amess, B., Townsend, R. R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M. J., Stein, R. C., Waterfield, M. D., Davies, S. C., and O'Hare, M. J. (1999) Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammoplasties. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12589–12594
2. Unlu, M., Morgan, M. E., and Minden, J. S. (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* **18,** 2071–2077
3. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., and Davison, M. (2001) Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* **1,** 377–396
4. Harris, R. A., Eichholtz, T. J., Hiles, I. D., Page, M. J., and O'Hare, M. J. (1999) New model of ErbB-2 over-expression in human mammary luminal epithelial cells. *Int. J. Cancer* **80,** 477–484
5. Stamps, A. C., Davies, S. C., Burman, J., and O'Hare, M. J. (1994) Analysis of proviral integration in human mammary epithelial cell lines immortalized by retroviral infection with a temperature-sensitive SV40 T-antigen construct. *Int. J. Cancer* **57,** p. 865–74
6. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68,** 850–858
7. Wilkins, M. R., Hochstrasser, D. F., Sanchez, J. C., Bairoch, A., and Appel R. D. (1996) Integrating two-dimensional gel databases using the Melanie II software. *Trends Biochem. Sci.* **21,** 496–497
8. Sarto, C., Binz, P. A., and Mocarelli, P. (2000) Heat shock proteins in human cancer. *Electrophoresis* **21,** 1218–1226
9. Lin, C. S., Aebersold, R. H., Kent, S. B., Varma, M., and Leavitt, J. (1988) Molecular cloning and characterization of plastin, a human leukocyte protein expressed in transformed human fibroblasts. *Mol. Cell. Biol.* **8,** 4659–4668
10. Lapillonne, A., Coue, O., Friederich, E., Nicolas, A., Del Maestro, L., Louvard, D., Robine, S., and Sastre-Garau, X. (2000) Expression patterns of L-plastin isoform in normal and carcinomatous breast tissues. *Anticancer Res.* **20,** 3177–3182
11. Lobo, C., Ruiz-Bellido, M. A., Aledo, J. C., Marquez, J., Nunez De Castro, I., and Alonso, F. J. (2000) Inhibition of glutaminase expression by antisense mRNA decreases growth and tumorigenicity of tumor cells. *Biochem. J.* **348,** 257–261
12. Takihara, Y., Matusa, Y., and Hara, J. (2000) Role of the beta isoform of 14-3-3 proteins in cellular proliferation and oncogenic transformation. *Carcinogenesis* **21,** 2073–2077
13. Yarden, Y., and Sliwkowski, M. X. (2000) Untangling the ErbB signaling network. *Nat Rev. Mol. Cell. Biol.* **2,** 127–137
14. Caudell, E. G., Caudell, J. J., Tang, C. H., Yu, T. K., Frederick, M. J., and Grimm, E. A. (2000) Characterization of human copine III as a phosphoprotein with associated kinase activity. *Biochemistry* **39,** 13034–13043
15. Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J., and Cohen, J. (1998) The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from Paramecium to humans. *J. Biol. Chem.* **273,** 1393–1402