Multivariable Difference Gel Electrophoresis and Mass Spectrometry

A CASE STUDY ON TRANSFORMING GROWTH FACTOR-β AND ERBB2 SIGNALING

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Multivariable DIGE/MS was used to investigate proteins altered in expression and/or post-translational modification in response to activation of transforming growth factor (TGF)-β receptors in MCF10A mammary epithelial cells overexpressing the HER2/Neu (ErbB2) oncogene. Proteome changes were monitored in response to exogenous TGF-β over time (0, 8, 24, and 40 h), and proteins were resolved using medium range (pH 4–7) and narrow range (pH 5.3–6.5) isoelectric focusing combined with up to 2 mg of protein to allow inspection of lower abundance proteins. Triplicate samples were prepared independently and analyzed together across multiple DIGE gels using a pooled sample internal standard to quantify expression changes with statistical confidence. Unsupervised principle component analysis and hierarchical clustering of the individual DIGE proteome expression maps provided independent confirmation of distinct expression patterns from the individual experiments and demonstrated high reproducibility between replicate samples. Fifty-nine proteins (including some isoforms) that exhibited significant kinetic expression changes were identified using mass spectrometry and database interrogation and were mapped to existing biological networks involved in TGF-β signaling. Several proteins with a potential role in breast cancer, such as maspin and cathepsin D, were identified as novel molecules associated with TGF-β signaling. Molecular & Cellular Proteomics 6:150–169, 2007.

2D¹ gel-based approaches are often used to survey the proteome on a global scale, typically resolving thousands of intact proteins based on charge (using isoelectric focusing) and apparent molecular mass (using SDS-PAGE). Despite its popularity for differential display proteomics, 2D gel-based strategies have until recently lacked the ability to directly quantify abundance changes in the same fashion as in stable isotope strategies using liquid chromatography coupled with tandem mass spectrometry (1–3), i.e. by multiplexing samples into a single run to remove analytical (gel-to-gel or column-to-column) variation. Multiplexing samples labeled with stable isotopes have been used in gel-based proteomics (4), but in this case abundance changes are monitored during the mass spectrometry stage on each individual protein prior to the knowledge of which proteins are changing.

DIGE technology (first described by Unlu et al. (5)) adds an essential quantitative component to 2D gel-based strategies and allows for the detection of subtle changes in protein abundance with statistical confidence (6–8). DIGE uses three spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) to label up to three samples to be run together on the same 2D gel. A pooled mixture containing an equal aliquot of all samples is made and labeled in bulk with Cy2 and used as an internal standard to coordinate between multiple DIGE gels with each gel containing two samples from the experiment that have been individually labeled with Cy3 or Cy5. This use of a pooled sample internal standard provides every resolved protein form under survey with a unique internal standard across a coordinated set of DIGE gels (Refs. 9 and 10; for a review, see Ref. 11) and allows for the quantitative comparison of proteomic changes with statistical confidence afforded by analyzing replicate samples relative to the same internal standard.

As with any complex system, pre- and postfractionation allows for access to lower abundance proteins by increasing the total amount of protein analyzed without overloading the analytical system. Prefractionation is useful for subproteomes but can often introduce additional non-biological variation into the samples that must be controlled. Postfractionation of complex samples is perhaps most popular in complementary peptide-based proteomics strategies using multidimensional HPLC separations prior to mass spectrometry (e.g. multidimensional protein identification technology (MudPIT) (12)). In a similar fashion, resolution and sensitivity can be improved in a 2D gel experiment by postfractonating complex proteomes with medium range (e.g. pH 4–7) and narrow range (e.g. pH...
5.3–6.5) isoelectric focusing gradients with commensurate increases in protein load (13). By resolving intact proteins, 2D gels can resolve multiply charged isoforms (that may result from phosphorylation or other charged post-translational modifications) and biologically significant proteolytic products. Subsequent mass spectrometry can verify that a set of isoforms is in fact related without necessarily identifying the modified peptide(s), whereas such changes may be completely overlooked in the more sensitive peptide-based approaches without mass spectral information on the modified peptide(s).

The main objective of this study was to demonstrate the technical advantage of DIGE/MS to facely quantify changes in protein abundance and/or post-translational modification on a global scale from multiple experimental variables, each with independent biological repetition. To this end, we used as a model system transforming growth factor-β (TGF-β) stimulation of human mammary epithelial cells transfected with a HER2 expression vector. This model system is of particular biological interest because overexpression of the tyrosine kinase receptor HER2/Neu (ErbB2) is detected in ~25% of breast cancers (14). TGF-β is a cytokine that suppresses early tumor formation but promotes tumor progression and metastasis at later stages (15) and has been shown to synergize with ErbB receptor tyrosine kinases. For example, overexpression of active TGF-β1 (or active receptor mutants) in transgenic mice also expressing murine mammary tumor virus/Neu (ErbB2) accelerates metastases from Neu-induced mammary cancers (16–18). A genetic modifier screen in non-tumorigenic mammary epithelial cells identified TGF-β1 and TGF-β3 as molecules that cooperate with HER2 in inducing cell motility and invasion (19), and inhibition of HER2 with the antibody trastuzumab blocked the promigratory effect of TGF-β on HER2-overexpressing mammary epithelial cells (20). Despite these and other studies, little is known regarding the molecular mechanisms or downstream effectors of cross-talk between TGF-β and ErbB receptor signaling, making the findings herein of potential value for further investigation.

Changes due to TGF-β stimulation were assessed over time (0, 8, 24, and 40 h) in triplicate experiments that were analyzed coordinately by DIGE. High resolution information on over 1500 protein forms was surveyed in the pH 4–7 range (0.5 mg of total protein per gel), and in some cases increased sensitivity and resolution were afforded by using narrow range isoelectric focusing (pH 5.3–6.5) in conjunction with up to 2 mg of total protein per gel. Principle component analysis (PCA) and unsupervised hierarchical clustering (HC) of the individual Cy3- and Cy5-labeled DIGE expression maps provided independent confirmation of distinct expression patterns from each group and demonstrated high reproducibility between the replicate samples. Each experimental condition was measured using independent replicates for statistical confidence and to rule out false-positive results due to non-biological variation. Proteins of interest were identified using mass spectrometry and database interrogation, and identified proteins were mapped to existing biological networks and pathways to reveal additional information regarding the relationship of the proteins identified in these studies.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Experimental Treatment, and Sample Preparation—** MCF10A/HER2 cells were generated and grown as described previously (20) in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 5% horse serum (HyClone), 20 ng/ml human recombinant epidermal growth factor (In Vitrogen), 100 ng/ml cholera toxin (Calbiochem), 10 μg/ml insulin (In Vitrogen), and 500 ng/ml hydrocortisone (Sigma).

Subconfluent MCF10A cells in 100-mm dishes were treated with culture medium supplemented with human recombinant TGF-β1 (R&D Systems) at a final concentration of 2 ng/ml at 0, 8, 24, and 40 h prior to harvesting. The cells were washed twice with ice-cold PBS and lysed with Nonidet P-40 Lysis Buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 20 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each aprotinin, pepstatin, and leupeptin). After sonication for 10 s and centrifugation (14,000 rpm), the protein concentration in the supernatants was measured using the bicinchoninic acid protein assay reagent (Pierce). Immunoblotting was performed as described previously (21). Horseradish peroxidase-conjugated secondary antibodies (Promega) were used for all immunoblots. Primary antibodies included: Smad2/3, hemagglutinin, maspin (Santa Cruz Biotechnology), P-Smad, P-MAPK, P-ErbB2/HER2 (Tyr-1248) (Cell Signaling, Technology, Inc., Beverly, MA); and cathepsin D and actin (Sigma).

**Extraction of Total RNA and RT-PCR—** Total RNA was extracted using the RNeasy minikit (Qiagen). RT-PCR was carried out using the Titanium one-step RT-PCR kit (BD Biosciences). For each RT-PCR, 100 ng of RNA were added to a 50-µl reaction system according to the manufacturer’s protocol (50 °C for 1 h followed by 30 cycles of PCR amplification) using primers 5′-gcaattgtagctcgtcagctacagcaatc and 5′-cacttgggagacaagaatggtcaaag specific for maspin cDNA. The PCR products were analyzed in 1.2% agarose gels.

**DIGE Experimental Design—** The mixed internal standard methodology of Friedman et al. (10) and Gerbasi et al. (22) was used with the following modifications. Each experiment contained four conditions repeated in triplicate, generating 12 individual samples that were co-resolved across six DIGE gels all coordinated by the same pooled sample internal standard. Experiments utilizing 24-cm pH 4–7 IEF gradients contained 0.5 mg of total protein equally divided between any two samples and an aliquot of the internal standard as follows. For each individual sample, 0.25 mg of protein was separately precipitated with methanol and chloroform (23) and resuspended in 30 µl of labeling buffer (7 M urea, 2 M thio urea, 4% CHAPS, 30 mM Tris, 5 mM magnesium acetate). One-third of each sample (10 µl, 83.3 μg) was removed and combined into a single tube to comprise the pooled sample internal standard. Thus for each six-gel experiment comprising 12 individual samples, the pooled standard contained 1000 μg. The remaining two-thirds of each individual sample (20 µl, 166.7 μg) was labeled with 200 pmol of either Cy3 or Cy5, whereas the pooled sample was labeled en masse with 1200 pmol of Cy2. A dye-swapping scheme was used such that the three samples from any condition were never labeled all with Cy3 or Cy5 to control for any dye-specific labeling artifacts that might occur. Experiments utilizing 24-cm pH 5.3–6.5 IEF gradients contained 2 mg of total protein treated essentially as above, only starting with 1 mg of extract from each sample, labeling individual samples with 400 pmol of Cy3/Cy5,
and labeling the pooled sample internal standard with 2400 pmol of Cy2.

The N-hydroxysuccinimidy1 ester forms of Cy2, Cy3, and Cy5 were used following standard methods for minimal labeling. Briefly labeling was performed for 30 min on ice in the dark after which the reactions were quenched with the addition of 10 mM lysine (2 μl for each 200 pmol of dye) for 10 min on ice in the dark. The quenched Cy3- and Cy5-labeled samples (each containing 166.7 μg of protein) were then combined and mixed with a 166.7-μg aliquot of the Cy2-labeled pooled standard after which an equal volume of 2× rehydration buffer (7 mM urea, 2 mM thiourea, 4% CHAPS, 4 mg/ml DTT) was added. The mixtures were brought up to final volume of 450 μl with 1× rehydration buffer (same as above except for 2 mg/ml DTT) after which 0.5% IPG buffer 4–7 or 5.3–6.5 was added and mixed thoroughly. Although the dye:sample ratios are skewed from the manufacturer’s recommendation, we have validated that these ratios provide sensitive labeling comparable to SYPRO Ruby total protein staining while allowing for optimal protein amounts to facilitate subsequent mass spectrometry (10, 22, 24–26).

For each six-gel DIGE experiment, tripartite-labeled samples for each gel (450 μl final volume) were passively rehydrated into 24-cm pH 4–7 and pH 5.3–6.5 IPG strips (Amersham Biosciences/GE Healthcare) for 24 h followed by simultaneous isoelectric focusing using a manifold-equipped IPGphor IEF unit (Amersham Biosciences/GE Healthcare) according to the manufacturer’s instructions for a total of 60 kV-h. The cysteine sulfhydrils were reduced and carbamidomethylated while the proteins were equilibrated into the second dimension loading buffer by incubating the focused strips in equilibration buffer (30% glycerol, 2% SDS, 6 M urea, 50 mM Tris, pH 8.8, 8 ppm bromophenol blue) supplemented with 1% DTT for 20 min at room temperature followed by 2.5% iodoacetamide in fresh equilibration buffer for an additional 20-min room temperature incubation. Second dimensional SDS-PAGE was performed on hand-cast 12% SDS-PAGE gels using low fluorescence glass plates with one plate presilanized to preferentially affix the gel, thereby ensuring the accuracy of subsequent robotic protein excision. Electrophoresis was carried out at 0.2 watts/gel for 3 h followed by 20 watts/gel until completion using a DALT-12 unit (Amersham Biosciences/GE Healthcare).

The differentially labeled co-resolved proteome maps within each DIGE gel were imaged at 100-μm resolution separately by dye-specific excitation and emission wavelengths using a Typhoon 9400 variable mode imager (Amersham Biosciences/GE Healthcare). 16-bit tagged image file format images were cropped and exported for analysis using the DeCyder version 6.5 suite of software tools. After imaging for CyDye components, the non-silanized glass plate was removed, and the gels were fixed in 50% methanol, 7% acetic acid for 2 h and then incubated in SYPRO Ruby (Invitrogen) in the dark overnight. This poststain also visualizes approximately 97% of unlabelled protein and ensures accurate protein excision as the molecular weight and hydrophobicity of the CyDyes influence the apparent molecular mass of proteins during SDS-PAGE. SYPRO Ruby images were acquired on the same imager as well as reimagined postexcision to ensure accurate protein excision.

**DIGE Analysis**—The DeCyder version 6.5 suite of software tools (Amersham Biosciences/GE Healthcare) was used for DIGE analysis. The Differential In-gel Analysis module was used to quantitatively compare the normalized volume ratio of each individual protein spot feature from a Cy3- or Cy5-labeled sample on a given gel relative to the Cy2 signal from the pooled sample internal standard corresponding to the same spot feature. Within each gel, the co-resolved fluorescent signals from each protein (two individual samples and one internal standard) are co-detected by the software, and abundance measurements are made directly to the internal standard without interference from gel-to-gel variation, obviating the need to run analytical replicates for each sample.

The Differential In-gel Analysis datasets for each individual gel were then collectively analyzed using the Biological Variation Analysis module, which allowed for the facile matching of protein migration patterns and normalization of Cy3/Cy2 and Cy5/Cy2 quantitative abundance ratios for each protein between gels of a coordinated set, again using the unique signal of each protein from the pooled internal standard. In this way, multiple variables each with independent experimental repetition were coordinately quantified with statistical confidence and without the requirement that every pairwise comparison be made within a single 2D DIGE gel. Statistical significance was associated with each change in abundance or charge-altering post-translational modification using Student’s t-test and analysis of variance (ANOVA) analyses that compare the variation of expression within a group to the magnitude of change between groups. Many statistically significant changes were observed within the 99.9th percentile confidence interval (representing less than one false positive for approximately 1500 proteins resolved in a gel), but changes within the 95th percentile were also considered.

Unsupervised PCA and HC was performed using the DeCyder Extended Data Analysis module. These multivariate analyses clustered the individual Cy3- and Cy5-labeled samples based on the collective comparison of expression patterns from the proteins identified in Table I. These groups of protein expression characteristics are represented by each data point in the PCA plots and by each column in the HC expression matrices (heat maps). PCA reduces the complexity of a multidimensional analysis into two principle components, PC1 and PC2, which orthogonally divide the samples based on the two largest sources of variation in the dataset. Values within the circles of the PCA plots are within the 95th percentile confidence interval. HC performs a similar unsupervised clustering of the samples based on similarities of expression patterns in the selected proteins, which are visually presented as horizontal lines in an expression matrix “heat map” using a standardized log abundance scale ranging from −0.5 (green) to +0.5 (red). HC expression matrices were calculated using Euclidean correlation and average linkage. Mapping of proteins identified by mass spectrometry and database interrogation (see below) onto existing networks and pathways was accomplished using Ingenuity Pathway Analysis software (Ingenuity Systems, Inc.).

**In-gel Digestion, Mass Spectrometry, and Database Interrogation**—Proteins of interest were robotically excised and digested into peptides in-gel with modified porcine trypsin protease (Trypsin Gold, Promega), and peptides were applied to a stainless steel target using an integrated Spot Handling Workstation (Amersham Biosciences/GE Healthcare) according to the manufacturer’s recommendations. Peptide samples (0.3 μl) were robotically mixed wet on the target with an equal volume of α-cyano-4-hydroxycinnamic acid (5 mg/ml in 60% acetonitrile, 0.1% trifluoroacetic acid supplemented with 1 mg/ml ammonium citrate).

MALDI-TOF MS and data-dependent TOF/TOF tandem MS/MS was performed on a Voyager 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 1500 laser shots per spectrum. Peptide ion masses (M + H) were accurate to within 20 ppm after internal calibration using the trypsin autolytic peptides at m/z 842.51 and 2211.10. TOF/TOF tandem MS fragmentation spectra were acquired in a data-dependent fashion based on the MALDI-TOF peptide mass map for each protein, averaging 2000 laser shots per fragmentation spectrum on each of the 20 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

The resulting peptide mass maps and the associated fragmentation spectra were collectively used to interrogate sequences present...
in the Swiss-Prot and National Center for Biotechnology Information non-redundant (NCBInr) databases to generate statistically significant candidate identifications using GPS Explorer software (Applied Bio-systems) running the MASCOT search algorithm (Matrix Science). Searches were performed without constraining protein molecular weight or isoelectric point, with complete carbamidomethylation of cysteine, with partial oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Evidence of proteins derived from mycoplasma was found in the course of these studies but did not have an effect on the statistical significance of the proteins presented in this study. Significant Molecular Weight Search (MOWSE) scores (p < 0.05), number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region with predicted molecular weight and pl were collectively considered for each protein identification (all data are presented in Table I and Supplemental Table 1).

RESULTS

Time Course of TGF-β-induced Proteome Changes Measured by DIGE/MS—MCF10A/HER2 cells were treated with 2 ng/ml TGF-β1 for 0, 8, 24, and 40 h, and efficacy of ligand treatment was confirmed by monitoring phosphorylation of Smad2, which occurs directly by the TGF-β type I receptor upon ligand binding (Fig. 1A). To assess proteomic changes in these samples, we performed DIGE/MS analysis using a pooled sample internal standard present on every gel (see "Experimental Procedures"). With this experimental design, every DIGE gel in a six-gel set contains two of the 12 samples (three replicates from four time points) along with an equal aliquot of the Cy2-labeled internal standard (std). Within the boxed regions representing each labeled sample is depicted a theoretical protein that is up-regulated at the 40-h time point. Dotted lines illustrate how the protein signals from each sample are directly quantified relative to the Cy2 internal standard signal for that protein without interference from gel-to-gel variation and how the Cy3:Cy2 and Cy5:Cy2 intragel ratios are normalized between the six gels. D, a graphical representation of the normalized abundance ratios for this theoretical protein change.
A schematic of the loading matrix, indicating how the individual samples were labeled and loaded into the six DIGE gels along with the internal standard, is shown in Fig. 1C. Within this loading matrix, we have depicted a theoretical protein that is up-regulated at the 40-h time point and indicated (with dotted lines), how the relative abundance is quantified for this protein within each gel relative to the cognate signal from the protein in the Cy2-labeled internal standard (without gel-to-gel variation), and how the Cy3:CY2 and Cy5:CY2 ratios are then normalized between the gels using the CY2 signal for that protein. The graphical readout for this theoretical change is shown in Fig. 1D where the normalized abundance ratios (i.e., relative to the internal standard that is normalized across all six gels) are all graphed together to easily visualize the relative magnitude and reproducibility of the expression data.

DIGE analysis flagged 26 protein forms that exhibited statistically significant (ANOVA, n = 3) changes in abundance or charge-altering post-translational modification that were greater than 1.2-fold displaying early, late, or biphasic kinetics (Fig. 2A and Table I, lines 1–26). Many of the changes fell within the 99.9th percentile confidence interval where only one false positive is expected for over 1000 features, but changes within the 95th percentile were also considered (Table I, lines 1–26).

Proteins were excised from a subset of the six gels and subjected to protein identification using mass spectrometry and database interrogation as described under “Experimental Procedures,” the results of which are summarized in Table I (lines 1–26) and detailed in Supplemental Table 1. The 26 identified features specified 23 unique proteins including redundancies due to post-translational modification or proteolysis. Many of these 23 proteins were also found as additional charge-related isoforms (migrating with closely related isoelectric points but indistinguishable apparent molecular masses, data not shown) consistent with a charge-altering post-translational modification. However, the expression patterns of these isoforms were overall similar to those listed in Table I, and in most cases these were omitted from Table I for brevity.

The above analysis used medium range pH 4–7 gradients for the first dimension isoelectric focusing because this condition offers increased resolution and sensitivity (loading 0.5 mg of total protein into each gel) compared with broader range gradients (e.g., pH 3–11) (13). To increase further the pI resolution and to gain access to lower abundance proteins in these samples, we reanalyzed the same samples using narrow range DIGE analysis exhibiting kinetics similar to those observed above that were subsequently identified by mass spectrometry and database interrogation (Table I and Supplemental Table 1, lines 27–59). Comparison of the proteome maps and DIGE/MS results from the two pH range experiments revealed 11 new features displaying significant DIGE results in the narrow pH range that were either too low abundance to be detected by DIGE and/or subsequent mass spectrometry in the pH 4–7 experiment (features 30, 35, 40, 51, 58, and 59) or were poorly resolved in the pH 4–7 range (features 28, 31, 36, 37, and 46). Two proteins identified in the pH 4–7 range, cathepsin D (basic isoform, feature 22) and Hsp27 (feature 24), were reconfirmed by mass spectrometry in the narrow range experiment to demonstrate consistency in DIGE results between the two analyses (Table I, lines 22, 24, 53, and 57). Eight additional proteins in the pH 4–7 range (features 4–6, 12–14, 20, and 26) also resolved in the narrow range gels and were
similarly confirmed by positional matching and inspection of the DIGE profiles but were not further confirmed by mass spectrometry (data not shown). The remaining 20 features listed in Table I for the narrow range experiment displayed only marginal changes (in magnitude and/or statistical significance) that may not have been selected in the first pH 4–7 experiment due to the presence of stronger candidates in the dataset.

**Principle Component Analysis, Hierarchical Clustering, and Pathway Analysis**—We sought to further validate the experimental samples for relevancy of ligand binding and to establish biological significance of the resulting protein changes (as opposed to stochastic changes) by performing multivariate statistical tests and network mapping of the proteins identified by DIGE/MS. PCA reduces the dimensionality of a multidimensional analysis to display the two principle components that distinguish between the two largest sources of variation within the dataset.

In both pH range analyses, PCA indicated distinct expression patterns from the four groups and demonstrated high reproducibility between the replicate samples (Fig. 3, A and B). Each data point in the PCA plots describes the collective expression profiles for the subset of proteins listed in Table I for each pH range (Fig. 3, A and B). For the 26 features identified in the pH 4–7 analysis, the first principle component distinguished 65.5% of the variance with 19.4% additional variation distinguished by the second principle component. For the pH 5.3–6.5 analysis, these values were 81.1% of the variance and 8.3% additional variance for PC1 and PC2, respectively. In addition, the PCAs demonstrate that the greatest amount of variation in the experiment is what distinguishes the 40-h time point from the others. Although quite similar, the different relative orientations of the groups between the pH 4–7 and 5.3–6.5 analyses are most likely due to a different subset of proteins used for the analysis in each pH range.

These grouping assignments were reiterated in an unsupervised HC analysis of the protein expression patterns within each sample (Fig. 3, C and D). HC compares groups based on similarity of the collective expression patterns of the selected proteins with similarity being proportional to the lateral distance depicted in the branched dendograms above each expression matrix (heat map). Each column in the HC expression matrix is effectively the same as each data point in the PCA plots. For clarity, expression and identification information for each of the individual proteins in the HC analysis (grouped into horizontal bars in the expression matrix and related via a similar dendrogram on the left) is directly tied to the numerical line entry in Table I, which summarizes the mass spectrometry search results and details the DIGE results. Similar PCA and HC results were found for both pH ranges when expanding the dataset to over 100 features by relaxing significance thresholds (data not shown). The PCA and HC results validate the biological significance of the protein expression changes detailed in Table I as we would not expect these individual samples to cluster in this way if these individual changes arose stochastically.

In a similar fashion, we sought to validate the significance of the proteins listed in Table I by mapping them to pre-existing mammalian networks and pathways (Fig. 4 and Supplemental Table 2). Of the 51 unique proteins specified by the 59 protein forms identified in the pH 4–7 and pH 5.3–6.5 TGF-β time course experiments, 46 mapped to a network of pathways involving TGF-β1 as a major hub (Fig. 4). Associated with this network were intercalating pathways involving MYC, p53, and the peroxisome proliferative-activated receptor-α (PPARα) that in turn affected many proteins that were independently identified in the DIGE experiments (Fig. 4, shaded proteins). Although additional validation is necessary to establish biological significance, the mapping of these proteins to established networks also provides new insight into potential TGF-β effectors and pathways that might otherwise have gone unnoticed based solely on the list of proteins presented in Table I.

**Maspin and Cathepsin D**—Several proteins identified by DIGE/MS as potential new TGF-β effectors had demonstrated roles in breast cancer. Maspin, a tumor-suppressing serpin (serine protease inhibitor; Table I, lines 13 and 14, highlighted in Fig. 5, A–D), is expressed in non-tumor mammary epithelial cells but not in most human breast cancer cell lines or primary breast tumors (27). Restoration of maspin expression or treatment with recombinant maspin protein in MDA-MB-435 and MDA-MB-231 cancer cells reduces Rac1 activity and cell invasiveness as well as metastases in nude mice (27, 28). We found two isoforms of maspin that were increasing 1.65–1.67-fold (65–67% increase, p = 0.003 and 0.0069, respectively) after 40 h of treatment with TGF-β.

These findings were validated by Western and RT-PCR analyses using a new time course of TGF-β-induced maspin expression with or without concomitant overexpression of HER2 (Fig. 6). Maspin protein levels were up-regulated by exogenous TGF-β in both HER2-overexpressing cells and controls (Fig. 6A), implying that this effect does not depend on high levels of HER2. TGF-β treatment induced similar increases in maspin mRNA levels, indicating that the level of regulation was transcriptional (Fig. 6B).

In another example, two charge-related isoforms of cathepsin D, a lysosomal aspartyl endopeptidase, were altered by treatment with TGF-β (features 21, 22, and 53; highlighted in Fig. 5, E–H). In this case, both the magnitude and direction of the change differed between the two isoforms of cathepsin D, indicating a change in a charge-altering modification. At 40 h after treatment, the acidic isoform (feature 21) decreased by 34% (1.51-fold decrease, p = 0.021), whereas the basic isoform increased by as much as 195%, or 2.95-fold (p = 0.0000017; Table I, lines 22 and 53).

MS and database interrogation unambiguously identified both isoforms as cathepsin D despite low protein expression
### Table I

**TGFβ time course, pH 4–7 and pH 5.3–6.5**

HMG, 3-hydroxy-3-methylglutaryl.

| Pos. | Protein Identification | Accession Number | M.W. (kDa) | MOB112 | DENS | AV. Ratio | T-test | AV. Ratio | T-test | AV. Ratio | T-test | AV. Ratio | T-test | 1-ANOVA |
|------|------------------------|------------------|------------|--------|------|----------|--------|----------|--------|----------|--------|----------|--------|---------|
| 1    | Cog-70 oxygen-regulated protein | Q09441.1         | 111.9      | 5.2    | 122.9 | 0.023    | 1.05   | 0.48     | 1.58   | 0.0024   | 1.41   | 0.023    | 1.5    | 0.0005  |
| 2    | Integrin-β8             | P39356.1         | 123.6      | 5.5    | 98.8  | 1.15     | -1.07  | 0.39     | 1.2    | 0.099    | 1.57   | 0.0048   | 1.28   | 0.957   |
| 3    | Heat shock 70kD         | Q02584.1         | 96.9       | 5.4    | 96.9  | 2.13     | 1.07   | 0.45     | -1.02  | 0.7      | -1.57  | 0.0064   | -1.69  | 0.37    |
| 4    | MBH2 DNA-mismatch repair | P48273.1        | 105.4      | 5.8    | 95.7  | 2.24     | -1.07  | 0.7      | -1.24  | 0.10     | -1.4   | 0.041    | -1.17  | 0.34    |
| 5    | MBH2 DNA-mismatch repair | P48274.1        | 105.4      | 5.8    | 95.7  | 2.24     | -1.07  | 0.7      | -1.24  | 0.10     | -1.4   | 0.041    | -1.17  | 0.34    |
| 6    | Osmotic stress protein 54 (heat shock 70kD-related protein HSP-70) | Q96577.1   | 96.9      | 5.4    | 96.9  | 2.13     | 1.06   | 0.48     | -1.21  | 0.068    | -1.29  | 0.0092   | -1.29  | 0.934   |
| 7    | MADD1-related proapoptosis (TAD-domain-containing) | P39371.1   | 60.4      | 13.2   | 84.1  | 0.332    | 1.01   | 0.84     | 1      | 0.96      | 1.3    | 0.0088   | -1.61  | 0.01    |
| 8    | Prolyl hydroxylase alpha-2 subunit | P15420.1   | 81.4      | 5.5    | 89.6  | 2.17     | 1.08   | 0.58     | 1.33   | 0.011     | 1.59   | 0.0087   | 1.24   | 0.0058  |
| 9    | HMG-CoA synthase         | G0159R1         | 56.4      | 5.2    | 60.6  | 2.3%     | 1.22   | 0.02     | -1.19  | 0.056     | -2.36  | 0.0001   | -1.46  | 0.0024  |
| 10   | Cytokeratin 14           | P26253.1        | 82.4      | 5.1    | 135.19 | 1.68%    | 1.13   | 0.16     | 1.48   | 0.0044    | 1.8    | 0.002    | 1.3    | 0.0034  |
| 11   | Cell organelle protein   | Q69064.1        | 47K5      | 5.3    | 317.12 | 3.32%    | 1.07   | 0.12     | 1.31   | 0.0019    | 1.32   | 0.0003   | 1.25   | 0.0028  |

**DIGE profile**
- 1 - 0 h (no treatment)
- 2 - 8 h
- 3 - 24 h
- 4 - 40 h
| Pos. | Protein Identification                  | Accession number | Mw, Da | PI | MOWSE | Av. Ratio | T. score | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | Av. Ratio | T. test | 1  | 2  | 3  | 4  |
|------|----------------------------------------|------------------|--------|----|-------|-----------|----------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|---|---|---|---|
| 12   | macrophage activating protein isoform-1 regulatory protein (CAP-G0) | P40724           | 30.844  | 5.3 | 117.3  | 5.2258 | 1.09     | 0.19     | 1.18     | 0.013     | 1.3     | 0.01     | 1.09     | 0.14     | 1.19     | 0.046     | 1.65     | 0.14     | 0.0053    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 13   | metap2                                 | P36527           | 45.540  | 5.7 | 46.01  | 5.3568 | 1.25     | 0.025    | 1.52     | 0.0078    | 1.65     | 0.003     | 1.21     | 0.0981   | 1.32     | 0.026     | 1.69     | 0.042     | 0.0012    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 14   | metap2                                 | P36527           | 45.540  | 5.7 | 46.01  | 5.3568 | 1.33     | 0.0042   | 1.63     | 0.0036    | 1.67     | 0.0069    | 1.23     | 0.0969   | 1.26     | 0.0069    | 1.63     | 0.085     | 0.0016    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 15   | lysophosphatidylcholine synthase         | P49224           | 41.42   | 5.1 | 78.04  | 5.2232 | 1.03     | 0.39     | -1.05    | 0.3       | -1.29    | 0.0038    | -1.68    | 0.065     | -1.32    | 0.0067    | -1.22    | 0.0092    | 0.0026    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 16   | tubulin-like protein                    | P49396           | 32.644  | 4.6 | 46.01  | 5.3568 | 1.09     | 0.059    | -1.09    | 0.043     | -1.29    | 0.0048    | -1.19    | 0.0004    | -1.4     | 0.0014    | -1.18    | 0.017     | 0.0011    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 17   | kinesin TO alpha subunit                | P09405           | 32.742  | 4.7 | 103.5  | 6.3158 | 2.25     | 0.026    | 3.80     | 0.0044    | 2.52     | 0.612     | 1.73     | 0.0069    | 1.3      | 0.14       | -1.35    | 0.072     | 0.0069    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 18   | profilin I and II coiled-coil domain (pcdn) | P10694           | 29.142  | 4.4 | 31.32  | 5.0314 | 1.09     | 0.33     | -1.09    | 0.44      | -1.72    | 0.023     | -1.19    | 0.1       | -1.87    | 0.0004    | -1.58    | 0.0063    | 0.0029    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 19   | annexin V                              | P56758           | 35.842  | 4.4 | 93.62  | 5.0520 | 1.06     | 0.64     | 1.41     | 0.0021    | 1.42     | 0.0064    | 1.3      | 0.023     | 1.34     | 0.0024     | 1.61     | 0.04       | 0.0022    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 20   | annexin A                              | P08275           | 35.842  | 5.4 | 160.11  | 5.3313 | 1.01     | 0.68     | 1.23     | 0.024     | 1.45     | 0.0088    | 1.22     | 0.032     | 1.43     | 0.011      | 1.18     | 0.11       | 0.0032    |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 21   | cathepsin O                            | P00999           | 31.67   | 5.5 | 111.62 | 5.1688 | 1.04     | 0.78     | 1.01     | 0.94      | -1.52    | 0.0398    | -1.03    | 0.81      | -1.58    | 0.0275     | -1.52    | 0.0255     | 0.021     |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 22   | cathepsin O                            | P00999           | 31.67   | 5.5 | 111.62 | 5.1688 | 1.01     | 0.94     | 1.1      | 0.3       | 2.65     | 0.00010   | 1.09     | 0.31      | 2.03     | 0.00017    | 2.69     | 9.00E-06    | 1.70E-06  |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
| 23   | mGluR3 carboxy terminal protease/3C-like | P40724           | 30.844  | 5.3 | 110.51 | 5.2232 | -1.04    | 0.36     | 1.14     | 0.219     | 1.4      | 0.0025    | 1.19     | 0.0011    | 1.45     | 0.00089    | 1.22     | 0.0082     | 3.80E-05  |          |          |          |          |          |          |          | 1  | 2  | 3  | 4  |
### Table I—continued

#### 0.5 mg pH 4.7

| Pos. | protein identification | Accession number | MOLSCC | Dige profile | 1 | 2 | 3 | 4 |
|------|------------------------|------------------|--------|--------------|---|---|---|---|
| 24   | Hsp70                  | P04792           | 22.6±0.5 0.1 μl 140.1,102.7,76% | 2.2 | 0.0002 1.99 0.006 4.43 7.98E-05 1.3 0.12 2.01 0.001 2.51 6.0054 1.20E-05 |
| 25   | KRT16B protein (kertin fragment) | P05289 | 28.4±0.4 0.1 μl 168.1±2.3,176% | 2.2 | 0.0002 1.18 0.42 2.02 0.0024 0.187 0.0049 4.45 2.10E-05 2.38 6.0056 1.40E-05 |

#### 2 mg, pH 5.3-6.5

| Pos. | protein identification | Accession number | MOLSCC | Dige profile | 1 | 2 | 3 | 4 |
|------|------------------------|------------------|--------|--------------|---|---|---|---|
| 27   | glycyl tRNA-synthetase | P03280           | 84.4±0.1 0.1 μl 245.1098,4.35 | 1 | 0.51 0.92 0.32 1.18 0.0013 0.03 0.47 0.17 0.007 1.1 6.0055 1.0012 |
| 28   | hsp70 tumor necrosis factor receptor associated protein (TRAP) | Q15243 | 28.4±0.4 0.1 μl 212.1900,7.16 | 0.01 0.86 0.19 0.0016 0.13 0.0027 0.21 0.013 0.05 0.106 0.23 0.0049 |
| 29   | T-complex protein 1 gamma (chaperones) | P03280 | 61.4±0.4 0.1 μl 75.0±1.2,36 | 0.04 0.57 0.07 0.17 0.012 0.12 0.21 0.22 0.044 1.09 0.15 0.04 |
| 30   | olfactomedin-related protein 2 | Q15355 | 83.4±0.4 0.1 μl 363.1900,8.4 | -1.1 0.08 -1.36 0.011 -2.7 0.0044 1.24 0.057 2.45 0.0043 1.16 0.0051 1.00E-05 |
| 31   | lamin A/C | P03435 | 74.4±0.4 0.1 μl 111.7,106,1.19 | -1.06 0.03 -1.22 0.073 1.83 0.0028 1.16 0.19 1.73 0.0023 1.5 0.0073 0.0029 |
| 32   | stress-induced phosphoprotein 1B | P15484 | 83.4±0.4 0.1 μl 132.1629,3.29 | 1.04 0.45 -1.32 0.051 -1.31 0.004 1.17 0.057 1.37 0.0043 1.17 0.106 0.0015 |
| 33   | T-complex alpha | P17287 | 87.4±0.4 0.1 μl 522.2103,6.38 | -1.15 0.17 -1.32 0.051 -1.57 0.028 1.15 0.04 1.36 0.11 1.19 0.34 0.039 |
| Pos. | Protein Identification                          | Accession number | MMW (kDa) | MOWSE* | Av. Ratio | T-Test | Av. Ratio | T-Test | Av. Ratio | T-Test | Av. Ratio | T-Test | Av. Ratio | T-Test | Av. Ratio | T-Test | Av. Ratio | T-Test | Avg. Vol% |
|------|-----------------------------------------------|------------------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|
| 34   | UDP-N-acetyl-α-threonylcarboxypeptidase         | Q16522           | 50.4     | 6.97   | 102.1   | 0.012  | -0.66    | 0.205  | -1.29    | 0.053  | -1.05    | 0.17  | -1.31    | 0.046  | -1.25    | 0.968  | 0.014     |
| 35   | UDP-N-acetyl-α-threonylcarboxypeptidase         | Q16522           | 50.4     | 6.97   | 102.1   | 0.012  | -0.66    | 0.205  | -1.29    | 0.053  | -1.05    | 0.17  | -1.31    | 0.046  | -1.25    | 0.968  | 0.014     |
| 36   | Proteinase                                    | Q94926           | 85.4     | 4.21   | 192.1   | 0.25   | 0.0023   | 1.49   | 0.0015   | 1.34   | 0.026   | 1.19  | 0.0009   | 1.01   | 0.84     | -1.2   | 0.031     |
| 37   | Inosine-6-phosphate dehydrogenase 2            | P12995           | 35.5     | 4.4    | 72.5    | 0.09   | 0.23     | 1.15   | 0.15     | 1.39   | 0.1     | -1.27 | 0.0058   | -1.54  | 0.042    | -1.21  | 0.25      |
| 38   | FK506-binding protein 5                       | Q13051           | 32.5     | 5.7    | 66.6    | 0.09   | 0.23     | 1.15   | 0.15     | 1.39   | 0.1     | -1.27 | 0.0058   | -1.54  | 0.042    | -1.21  | 0.25      |
| 39   | T-complex beta                                | P19971           | 59.4     | 6.97   | 124.9   | 0.09   | 0.9      | 0.05   | 0.28     | 0.015  | -1.16    | 0.046  | -1.52    | 0.042  | -1.21    | 0.608  | 0.0043    |
| 40   | Ribosome                                    | Q67903           | 60.5     | 6.8    | 121.1   | 0.09   | 0.25     | 1.4    | 0.0009   | 1.04   | 0.49    | 1.36  | 0.0061   | 1.42   | 0.00068  | 0.00021|
| 41   | Ribosomal protein 1 (ribosomal protein 1)    | Q94926           | 85.4     | 4.21   | 192.1   | 0.25   | 0.0023   | 1.49   | 0.0015   | 1.34   | 0.026   | 1.19  | 0.0009   | 1.01   | 0.84     | -1.2   | 0.031     |
| 42   | Ribosomal protein 3 (ribosomal protein 3)    | P00018           | 48.5     | 5.6    | 105.9   | 0.14   | 0.0365   | 1.18   | 0.0021   | 1.08   | 0.19    | 1.14  | 0.015    | 1.05   | 0.28      |
| 43   | Ribosomal protein 4 (ribosomal protein 4)    | P00018           | 48.5     | 5.6    | 105.9   | 0.14   | 0.0365   | 1.18   | 0.0021   | 1.08   | 0.19    | 1.14  | 0.015    | 1.05   | 0.28      |
| 44   | Ribosomal protein 5 (ribosomal protein 5)    | P00018           | 48.5     | 5.6    | 105.9   | 0.14   | 0.0365   | 1.18   | 0.0021   | 1.08   | 0.19    | 1.14  | 0.015    | 1.05   | 0.28      |
| 45   | Ribosomal protein 6 (ribosomal protein 6)    | P00018           | 48.5     | 5.6    | 105.9   | 0.14   | 0.0365   | 1.18   | 0.0021   | 1.08   | 0.19    | 1.14  | 0.015    | 1.05   | 0.28      |
### Table I—continued

0.5 mg pH 4-7

| Pos. | protein identification | Accession number | MW (kDa) | pI | MOWSE* | Av. Ratio | T-test | Av. Ratio | T-test | Av. Ratio | T-test | Av. Ratio | T-test | Av. Ratio | T-test | Av. Ratio | T-test | Av. Ratio | T-test | 1-KNOX* |
|------|------------------------|------------------|----------|----|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|-----------|--------|
| 45   | adenylate kinase synthase 2 | P36926 | 30.94±0.5, 0.1 pl | 62.7±0.7, 2.1 | 1.01 | 0.9 | -1.09 | 0.06 | -1.21 | 0.012 | -1.09 | 0.13 | -1.22 | 0.024 | -1.12 | 0.04 | 0.0074 |
| 47   | EF-Tu | P49911 | 45.3±0.5, 0.1 pl | 58.2±0.1, 6.5 | -1 | 0.97 | -1.13 | 0.0049 | -1.02 | 0.46 | -1.12 | 0.014 | -1.02 | 0.02 | 1.1 | 0.024 | 0.0059 |
| 48   | adenosylhomocysteinase | P06909 | 38.6±0.5, 0.1 pl | 19.7±0.1, 10 | 1.03 | 0.91 | 1.16 | 0.046 | -1.03 | 0.3 | -1.2 | 0.029 | -1.17 | 0.026 | 0.0077 |
| 49   | eukaryotic translation factor 3 subunit 1 | O15074 | 49.6±0.5, 0.1 pl | 171.1±0.3, 4.6 | 1.05 | 0.9 | -1.14 | 0.014 | -1.35 | 0.0031 | -1.19 | 0.053 | -1.41 | 0.009 | -1.19 | 0.028 |
| 50   | lactate dehydrogenase mature | P50213 | 37.0±0.5, 0.1 pl | 128.8±1.2, 18 | -1.04 | 0.45 | -1.21 | 0.0042 | -1.07 | 0.35 | -1.17 | 0.035 | -1.03 | 0.71 | 1.13 | 0.14 | 0.047 |
| 51   | transaldolase | P21787 | 38.0±0.5, 0.1 pl | 193.1±0.3, 27 | -1.04 | 0.63 | -1.25 | 0.025 | -1.36 | 0.0032 | -1.35 | 0.003 | -1.2 | 0.024 | -1.09 | 0.17 | 0.0056 |
| 52   | Inorganic pyrophosphatase | O15081 | 39.0±0.5, 0.1 pl | 59.9±1.2, 24 | -1.09 | 0.45 | -1.17 | 0.041 | -1.24 | 0.004 | -1.11 | 0.24 | -1.17 | 0.035 | -1.06 | 0.31 | 0.024 |
| 53   | GAPDH | P57399 | 31.0±0.5, 0.1 pl | 137.0±0.3, 21 | 1.13 | 0.14 | 1.1 | 0.31 | 2.72 | 4.86E-06 | -1.02 | 0.82 | 2.41 | 0.0002 | 2.46 | 0.0005 | 4.10E-06 |
| 54   | 40S ribosomal protein S1A-1947 | P21473 | 32.0±0.5, 0.1 pl | 134.1±0.3, 17 | -1.03 | 0.45 | -1.14 | 0.0024 | 1.05 | 0.44 | -1.11 | 0.015 | 1.08 | 0.29 | 1.21 | 0.031 | 0.02 |
| 55   | pleiotrophic factor 4 thioredoxin 16 beta subunit | O14459 | 29.0±0.5, 0.1 pl | 132.7±0.3, 23 | 1.01 | 0.91 | 1.04 | 0.52 | 1.17 | 0.012 | 1.02 | 0.7 | 1.16 | 0.03 | 1.13 | 0.023 | 0.0038 |
| 56   | euk-A coA hydrolase | P50004 | 26.0±0.5, 0.1 pl | 130.8±1.2, 29 | -1 | 0.98 | -1.1 | 0.05 | 1.12 | 0.017 | -1.1 | 0.083 | 1.12 | 0.027 | 1.23 | 0.007 | 0.0026 |
| 57   | Hsp67 | P44762 | 20.0±0.5, 0.1 pl | 69.9±1.8, 6.5 | 2 | 2.02E-05 | 1.10 | 0.0039 | 3.48 | 1.50E-05 | -1.09 | 0.70E-05 | 1.74 | 0.0046 | 2.03 | 3.00E-05 | 7.20E-09 |
| Pos. | Protein Identification | Accession Number | MW, pI | Molecular & Cellular Proteomics | 0h vs. 8h | 0h vs. 24h | 0h vs. 40h | 8h vs. 40h | 24h vs. 40h | 24h profile |
|------|------------------------|------------------|--------|-------------------------------|----------|-----------|----------|----------|-----------|------------|
| 58   | des(lys) deamidase     | P02016           | 43,305, 1.15 | -1.14 0.028                  | -1.55 0.021 | -4.85 7.09E-05 | -1.35 0.057 | -4.24 8.90E-05 | -3.14 0.0312 | 1.60E-06 |
| 59   | deamidase              | P02016           | 43,305, 1.15 | -1.04 0.070                  | 1.02 0.81  | -1.52 0.021 | 1.06 0.39 | -1.47 0.016 | -1.55 0.016 | 0.004      |

1 Swiss-Prot database was searched unless otherwise noted.
2 Theoretical molecular weight (MW) and isoelectric point (pI) are calculated from the database entries, which often contain precursor sequences not present in the mature form migrating on the gel.
3 MOWSE = A, B, C, D are MOWSE combined MS and MS/MS search scores, number of peptides matched (number of unmatched peptides), number of peptides with MS/MS data, and percent of the amino acids accounted for by the matching peptides (coverage). Molecular Weight Search (MOWSE) scores above 5.0 are within the 95th percentile confidence interval. Scores were calculated using the MASCOT v1.19 database search algorithms. Complete MS and database search results are provided in Supplementary Table 1.
4, 5, 6, average volume ratios, Student's t-test p-values, and analysis of variants (ANOVA) p-values calculated using DeCyder software version 6.5, utilizing the mixed-sample internal standard methodology as described in materials and methods.
7 DIGE graphical profiles generated using DeCyder software version 6.5. The unlabeled y-axes represent the normalized log abundance ratios relative to the cognate signals present in the internal standard specific for each protein shown. They are provided to illustrate the reproducibility and relative magnitude of the changes listed in this Table.
8 Manual PMF from weak signal, second-order confidence.
9 Lower MOWSE scores due to automated searching against precursor sequences present in database. Fragmentation data and predicted MW, pI of mature form are consistent with identified protein expectations.
10 No significant matches in Swiss-Prot database, or match was better to entry in NCBI database.
FIG. 3. Unsupervised multivariate analysis of DIGE/MS results. PCA reduces the dimensionality of a multidimensional analysis and displays the two principle components that can distinguish between the two largest sources of variation within the dataset. Each data point in the PCA plots describes the global expression values for the subset of proteins listed in Table I (26 proteins for pH 4–7 and 33 proteins for pH 5.3–6.5). A, principle component analysis discretely clustered the 12 individual Cy3- and Cy5-labeled DIGE expression maps into the four time treatment groups differentiated by two principle components: PC1, which distinguishes 65.5% of the total variance in the analysis, and
levels and associated weak signals in the mass spectrometer (Fig. 5, F–H, Table I, and Supplemental Table 1, lines 21, 22, and 53). Although there was no indication in the mass spectral data as to the nature or location of the charged modification, without mass spectral data on a modified peptide, this significant change in protein expression may well have been overlooked in standard peptide-based proteomics analyses (e.g., LC/MS/MS shotgun analysis with spectral counting or stable isotope labeling strategies).

These results were validated by Western analysis using an independent time course of TGF-β/H9252 treatment (Fig. 6C). The antibody used cannot distinguish between isoforms as we did with DIGE/MS. However, an overall increase in cathepsin D levels is predicted if both isoforms are considered collectively, and this increase is evident in the Western blot results. Treatment with TGF-β increased the mature 31-kDa isoform in MCF10A/HER2 cells but not in control MCF10A/vector cells, indicating that the observed changes in cathepsin D were dependent on high levels of HER2 signaling.

DISCUSSION

In this study we have shown the utility of the DIGE/MS approach for the quantitative analysis of over 1500 resolved proteins (including modified isoforms) across multiple conditions using TGF-β and HER2 signaling as a model system. Analyzing replicate samples enabled statistical confidence to be assigned for each measurement of altered protein expression over multiple variables. Multiplexing samples into the same gel separations along with a pooled sample internal standard allowed for these quantitative measurements to be made with statistical confidence across all samples without interference from gel-to-gel variation while significantly reducing the number of gels necessary compared with conventional 2D gel-based proteomics.

The expression patterns of the proteins identified in this study most likely work in concert to promote phenotypic PC2, which distinguishes an additional 19.4% of the variance. Related samples are encircled for illustrative proposes only. B, similar PCA plot shown for the pH 5.3–6.5 range analysis with PC1 and PC2 distinguishing 81.1% of the variance and 8.3% additional variance, respectively. C, unsupervised hierarchical clustering of the 12 independent samples based on the global expression patterns of 26 proteins in the pH 4–7 range that are detailed in Table I. Hierarchical clustering of individual samples is shown on top, and clustering of individual proteins is shown on the left with relative expression values displayed as an expression matrix (heat map) using a standardized log abundance scale ranging from −0.5 (green) to +0.5 (red). The gel number (1–6) and Cy3/5 dye labeling for each sample is listed below, and the protein identifications with corresponding line entries in Table I (lines 1–26) are listed along the right-hand side. D, similar expression matrix from an unsupervised hierarchical clustering analysis of 33 proteins from the pH 5.3–6.5 range that are also detailed in Table I (lines 27–59).
The unsupervised multivariate analyses (PCA and HC) indicated high reproducibility between replicate samples and demonstrated distinct expression patterns from the four experimental groups, making it unlikely that the abundance changes reported in Table I were occurring stochastically. In addition, mapping the proteins identified by DIGE/MS to previously characterized networks and pathways revealed new insight into the inter-relationships of these proteins as well as identified additional potential effectors that are members of these pathways but not identified in the DIGE/MS analysis due to a variety of reasons (e.g., low abundance, low molecular weight, or basic pI). MYC and PPARA are nuclear transcriptional regulators affecting the expression of target proteins governing cell proliferation and differentiation, adhesion, apoptosis, cell cycle progression, and inflammation responses. It was recently found that p53 promotes the activation of multiple TGF-β target genes during Xenopus embryonic development (29). A model has been proposed describing cooperation between p53 and Smads in TGF-β-mediated gene transcription where Smad2 enters into the nuclei and associates with Smad4 and specific cofactors to bind target sequences (30). Thus it is not surprising that many features depict proteins identified in the present study, whereas unshaded features depict additional members of these networks and pathways that were not detected by DIGE/MS. Full names and annotations for the proteins represented in this network are listed in Supplemental Table 2.

Fig. 4. Networks and pathways associated with proteins identified by DIGE/MS from the TGF-β time course study (both pH ranges combined, Table I). Ingenuity Pathway Analysis software (Ingenuity Systems, Inc.) was used to map identified proteins onto existing mammalian pathways and networks that associate proteins based on known protein-protein interactions, mRNA expression studies, and other biochemical interactions established in the literature. Shaded features depict proteins identified in the present study, whereas unshaded features depict additional members of these networks and pathways that were not detected by DIGE/MS. Full names and annotations for the proteins represented in this network are listed in Supplemental Table 2.
of the proteins identified by DIGE/MS were secondary effectors of the TGF-β pathway.

Several proteins identified in these studies are known targets or downstream effectors of TGF-β signaling. For example, proliferating cell nuclear antigen (PCNA), a cell proliferation marker, was down-regulated by TGF-β by 1.72-fold (42%, $p = 0.003$) at 40 h (Table I, line 18). This is consistent with the known proliferation suppressive function of TGF-β that is retained in MCF10A/HER2 cells (20). In another example, Hsp27, which was up-regulated in response to TGF-β by as much as 4.43-fold (343%, $p = 0.000075$) at 40 h (Table I, lines 24 and 57), mediates TGF-β-induced cell motility as demonstrated using small interfering RNA to block TGF-β-mediated cell invasion in human prostate cancer (31). TGF-β has also been shown to induce Hsp27 phosphorylation in osteoblast-like MC3T3-E1 cells (32). Determining whether the observed increases found in the current study result from specific phosphorylation or overall protein abundance will require further investigation.

Many novel TGF-β or HER2 effectors were also identified in these studies; several of these have demonstrated roles in breast cancer. One example is the tumor suppressor maspin (27), the expression of which has been shown to be regulated by the p53 tumor suppressor family (33, 34). Furthermore mutant p53 and aberrant cytosine methylation cooperate to silence maspin expression in cancer cells (35). This relationship between maspin (SERPINB5) and p53 is also reflected in the pathway/network map for TGF-β treatment (Fig. 4) where p53 is also shown to modulate the expression of lamin A/C (LMNA), PCNA, Hsp105 (HSPH1), and stress-induced phosphoprotein Hsp70 (STIP1), all of which were independently identified in the DIGE analysis (Table I, lines 3, 18, 31, and 32). We demonstrated that TGF-β-induced maspin expression was at the level of transcription (Fig. 6B), and subsequent experimentation indicates a direct role for p53 in maspin expression.2

In another example, cathepsin D, which is implicated in a number of cancers, including breast (Ref. 36, for a review, see Ref. 37), was identified as two isoforms that were differentially expressed, indicating a change in post-translational modification as well as an overall increase in expression (Fig. 5, E–H, and Fig. 6C). The observed apparent molecular mass and isoelectric point of the basic isoform (features 22 and 53) was consistent with the mature 31-kDa heavy chain that is produced after several rounds of post-translational processing (Ref. 38, predicted $pI = 5.5$). The substantial shift in $pI$ for the acidic isoform is consistent with the addition of a single negative charge or with the removal of approximately 10 amino acids from the C terminus as proposed to occur in the lysosome (38, 39). One interpretation of these results is that not only are cathepsin D expression levels increasing at the later time points but also that the protein is undergoing a differential modification/processing. An alternate interpretation is that although more cathepsin D is being expressed at the later time points no more is being modified/processed into the acidic isoform.

That this type of quantitative change in isoforms may well be overlooked in peptide-based strategies exemplifies the complementary nature of these two proteomics technologies. Any single proteomics strategy provides access only to a subset of the proteome under study, and each has unique strengths and weaknesses. Although DIGE provides statistically significant quantitative information from replicate samples on over 1000 intact proteins and modified isoforms in a single run, very hydrophobic proteins remain difficult to resolve, and proteins outside of the $pI$ and molecular weight ranges will not be surveyed. Using complementary technologies can also provide corroborative evidence. For example, a recent study of TGF-β treatment of human lung cancer cells using a global quantitative peptide-based profiling strategy (iTRAQ (isobaric tags for relative and absolute quantitation)) identified several of the proteins described here by DIGE/MS (tropomyosin, Hsp27, EF-Tu, and cofilin) and mapped proteins to similar networks that also spoke to a cellular program of adhesion/invasion known to be up-regulated by TGF-β in transformed cells (40).

Although complementary, an overriding limitation that is universal to all proteomics platforms for global scale experiments is the constraint imposed by abundant proteins that limits the total amount of sample extract that can be loaded into the analytical system without compromising resolution. This problem is most often addressed by postfractionation of the sample with commensurate higher protein loads. For DIGE/MS, this is accomplished using medium range (e.g. pH 4–7, 7–11) and narrow range gradients (e.g. pH 5.3–6.5) that offer greater resolving power and sensitivity (as demonstrated here). In this regard, pH 3–11 gradients offer the lowest resolution and are most biased toward the high abundance proteins despite being inclusive to a wide range of protein $pI$ values.

Newer technologies that couple in-solution isoelectric focusing with molecular weight separations hold great promise for resolving even higher protein loads to access lower abundance proteins while retaining the protein content of all pH fractions for subsequent analysis. Calcitonin and tumor necrosis factor α, estimated at levels below 0.1 ng/ml, were recently reported from human serum using this approach after major protein depletion (41). Utilization of the maleimide CyDyes for saturation labeling of cysteine sulfhydryls offers about a 10-fold increased sensitivity, although there are currently only two CyDyes with this chemistry, and generating usable signals in the mass spectrometer usually requires increased material. Combining these technology platforms to maximize their complementary nature while minimizing the.

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2 S. E. Wang, C. W. Whitwell, A. Narasanna, F. Y. Wu, D. B. Friedman, and C. L. Arteaga, submitted for publication.
FIG. 5. TGF-β signaling regulates maspin and cathepsin D proteins in HER2-overexpressing cells. A, SYPRO Ruby total protein stain image of a region in gel 4 indicating the position of the acidic maspin isoform (feature 13, arrow). The DIGE graphical expression profile is also shown enlarged from Table I to illustrate the normalized relative abundance change and consistency across the independent replicates for each time point. Expression data are reported as the average percent change of normalized volume ratios for the indicated condition relative to 0 h or control with associated Student’s t test p value (calculated using DeCyder version 6.5 software). Similar values were found for the basic isoform (Table I, line 14). B, MALDI-TOF mass spectrum of the trypsin-digested peptides derived from the protein excised from gel 4, feature 13 (arrow in A). The spectrum was internally calibrated to <20 ppm mass accuracy using the trypsin autolytic peptide ions at m/z 842.51 and 2211.10. The other labeled peptide ions (M + H) were used to generate statistically significant matches to maspin as indicated in Table I and Supplemental Table 1 (line 13). C, TOF/TOF tandem mass spectrum of the fragment ions of the peptide ion at m/z 1870.93. Fragment ions with the charge retained on the N terminus (b-ions, numbered left to right) and with charge retained on the C terminus (y-ions, numbered right to
are indicated using the displayed amino acid sequence of the predicted maspin peptide. D, the peptide ion at m/z 1653.81 was not matched to a predicted peptide from the database entry for human maspin, but the TOF/TOF fragmentation pattern was consistent with a predicted maspin peptide containing a missense mutation of Asp to Thr or Ile to Val, neither of which was reported in the existing database entry. E, SYPRO Ruby gel image of gel 4 indicating proteins excised for the identification of cathepsin D isoforms (acidic feature 21 and basic feature 22) that were changing in opposite directions relative to the 0-h samples as indicated. F, MALDI-TOF peptide mass map resulting from the in-gel digestion of the basic isoform feature 53 excised from gel 7, internally calibrated as above. Peptide signals used for the statistically significant identification of cathepsin D (CATD) are labeled and listed in Table I and Supplemental Table 1 (lines 21, 22, and 53). The ion at m/z 1045.57 is derived from trypsin autolysis, and the ions labeled Bg were background ions present in every spectrum. G and H mark two ions for which the TOF/TOF tandem MS/MS spectra are also shown. G, tandem TOF/TOF mass spectrum of the peptide ion at m/z 1462.66 with b- and y-ions annotated for the predicted amino acid sequence as described above. H, similarly annotated tandem TOF/TOF mass spectrum of the peptide ion at m/z 1601.83. Similar MS and MS/MS spectra were acquired for protein features 21 and 22 that were comparable both with respect to m/z values and relative ion intensity albeit with lower scores (Table I and Supplemental Table 1).
were treated with TGF-β cDNA. Gene-specific primers were used to amplify the full-length maspin to RT-PCR as indicated under "Experimental Procedures." Maspin individual weaknesses will undoubtedly be necessary to increase the scope and depth of quantitative differential display proteomics.

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REFERENCES

1. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. 17, 994–999

2. Mason, D. E., and Liebler, D. C. (2003) Quantitative analysis of modified proteins by LC-MS/MS of peptides labeled with phenyl isocyanate. J. Proteome Res. 2, 265–272

3. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khanovsky, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhas, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics 3, 1154–1169

4. Vogt, J. A., Schroer, K., Holzer, K., Hunzinger, C., Klemm, M., Biefang-Arndt, K., Schillo, S., Cahill, M. A., Schrattenholz, A., Matthes, H., and Stegmann, W. (2003) Protein abundance quantification in embryonic stem cells using incomplete metabolic labelling with 13C amino acids, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, and analysis of relative isotopologue abundances of peptides. Rapid Commun. Mass Spectrom. 17, 1273–1282

5. 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

6. Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pogran, 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

7. Von Eggeling, F., Gawriljuk, A., Fiedler, W., Ernst, G., Claussen, U., Klose, J., and Romer, I. (2001) Fluorescent dual colour 2D-protein gel electrophoresis for rapid detection of differences in protein pattern with standard image analysis software. Int. J. Mol. Med. 8, 373–377

8. Gade, D., Thierrmann, J., Markowsky, D., and Rabus, R. (2003) Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp. strain 1. J. Mol. Microbiol. Biotechnol. 5, 240–251

9. Alban, A., David, S. O., Bjorkest, L., Andersson, C., Sloge, E., Lewis, S., and Currie, I. (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3, 36–44

10. Friedman, D. B., Hill, S., Keller, J. W., Merchant, N. B., Levy, S. E., Coffey, R. J., and Caprioli, R. M. (2004) Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 4, 793–811

11. Lilly, K. S., and Friedman, D. B. (2004) All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev. Proteomics 1, 401–409

12. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. 19, 242–247

13. Gorg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W. (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21, 1037–1053

14. Yarden, Y., and Silwkowski, M. X. (2001) Untangling the ErbB signalling network. Nat. Rev. Mol. Cell. Biol. 2, 127–137

15. Wakefield, L. M., and Roberts, A. B. (2002) TGF-β signaling: positive and negative effects on tumorigenesis. Curr. Opin. Genet. Dev. 12, 22–29

16. Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J., and Massague, J. (2003) Transforming growth factor β signaling impair Neu-induced mammary
tumorigenesis while promoting pulmonary metastasis. Proc. Natl. Acad. Sci. U. S. A. 100, 8430–8435

17. Muraoka, R. S., Koh, Y., Roebuck, L. R., Sanders, M. E., Brantley-Sieders, D., Gorska, A. E., Moses, H. L., and Arteaga, C. L. (2003) Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor \( \beta \). Mol. Cell. Biol. 23, 8691–8703

18. Muraoka-Cook, R. S., Shin, I., Yi, J. Y., Easterly, E., Barcellos-Hoff, M. H., Yingling, J. M., Zent, R., and Arteaga, C. L. (2006) Activated type I TGF\( \beta \) receptor kinase enhances the survival of mammary epithelial cells and accelerates tumor progression. Oncogene 25, 3408–3423

19. Seton-Rogers, S. E., Lu, Y., Hines, L. M., Koundinya, M., LaBaer, J., Muthuswamy, S. K., and Brugge, J. S. (2004) Cooperation of the ErbB2 receptor and transforming growth factor \( \beta \) in induction of migration and invasion in mammary epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 101, 1257–1262

20. Ueda, Y., Wang, S., Dumont, N., Yi, J. Y., Koh, Y., and Arteaga, C. L. (2004) Overexpression of HER2 (erbB2) in human breast epithelial cells un-masks transforming growth factor \( \beta \)-induced cell motility. J. Biol. Chem. 279, 24505–24513

21. Dumont, N., Bakin, A. V., and Arteaga, C. L. (2003) Autocrine transforming growth factor-\( \beta \) signaling mediates Smad-independent motility in human cancer cells. J. Biol. Chem. 278, 3275–3285

22. Gerbasi, V. R., Weaver, C. M., Hill, S., Friedman, D. B., and Link, A. J. (2004) Yeast Asc1p and mammalian RACK1 are functionally orthologous core 40S ribosomal proteins that repress gene expression. Mol. Cell. Biol. 24, 8276–8287

23. Wessel, D., and Fluge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143

24. Zhang, Y. Q., Matthies, H. J., Mancuso, J., Andrews, H. K., Woodruff, E., III, Friedman, D., and Broadie, K. (2004) The Drosophila fragile X-related protein regulates axoneme differentiation during biosynthesis of the lysosomal enzyme cathepsin D in lysosomes in vitro. Mol. Endocrinol. 19, 683–697

25. Dey, S. K. (2005) Proteomic analysis identifies immunophilin FK506 binding protein 4 (FKBP52) as a downstream target of Hoxa10 in the perimplantation mouse uterus. Mol. Endocrinol. 19, 683–697

26. Zhang, Y. Q., Friedman, D. B., Wang, Z., Woodruff, E., III, Pan, L., O’Donnell, J., and Broadie, K. (2005) Protein expression profiling of the drosophila fragile X mutant brain reveals up-regulation of monoamine synthesis. Mol. Cell. Proteomics 4, 278–290

27. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. (1994) Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science 263, 526–529

28. Odero-Marah, V. A., Khalkhal-Ellis, Z., Chunchapong, J., Amir, S., Seftor, R. E., Seftor, E. A., and Hendrix, M. J. (2003) Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. Cancer Biol. Ther. 2, 398–403

29. Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbianco, C., and Piccolo, S. (2003) Links between tumor suppressors: p53 is required for TGF-\( \beta \) gene responses by cooperating with Smads. Cell 113, 301–314

30. Dupont, S., Zacchigna, L., Adorno, M., Soligo, S., Volpin, D., Piccolo, S., and Cordenonsi, M. (2004) Convergence of p53 and TGF-\( \beta \) signaling networks. Cancer Lett. 213, 129–138

31. Xu, L., Chen, S., and Bergan, R. C. (2006) MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. Oncogene 25, 2987–2998

32. Shibamanu, M., Kuroki, T., and Nose, K. (1992) Cell-cycle dependent phosphorylation of HSP29 by TGF \( \beta \)1 and H\( \beta \)2, in normal mouse osteoblastic cells (MC3T3-E1), but not in their ras-transformants. Biochem. Biophys. Res. Commun. 187, 1418–1425

33. Zou, Z., Gao, C., Nagaich, A. K., Connell, T., Saito, S., Moul, J. W., Seth, P., Appella, E., and Srivastava, S. (2000) p53 regulates the expression of the tumor suppressor gene maspin. J. Biol. Chem. 275, 6051–6054

34. Kim, S., Han, J., Kim, J., and Park, C. (2004) Maspin expression is trans-activated by p53 and is critical for the modulation of lung cancer progression. Cancer Res. 64, 6900–6905

35. Oshiro, M. M., Watts, G. S., Wozniak, R. J., Junk, D. J., Munoz-Rodriguez, J. L., Domann, F. E., and Futschler, B. W. (2003) Mutant p53 and aberrant cytosome methylation cooperate to silence gene expression. Oncogene 22, 3624–3634

36. Rochefort, H., Garcia, M., Glundau, M., Laurent, V., Liaudet, E., Rey, J. M., and Roger, P. (2000) Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. Clin. Chim. Acta 291, 157–170

37. Liaudet-Coopman, E., Beaujouin, M., Derozo, P., Garcia, M., Glundau-Lassis, M., Laurent-Matha, V., Prebois, C., Rochefort, H., and Vignon, F. (2005) Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. Cancer Lett. 18, 18

38. Gieselmann, V., Hasilik, A., and von Figura, K. (1985) Processing of human hemoglobin in vitro. J. Biol. Chem. 260, 3215–3220

39. Erickson, A. H., and Blobel, G. (1983) Carboxyl-terminal proteolytic processing during biosynthesis of the lysosomal enzymes \( \beta \)-glucuronidase and cathepsin D. Biochemistry 22, 5201–5205

40. Keshamouni, V. G., Michailidis, G., Grasso, C. S., Anthwal, S., Strahler, J. R., Walker, A., Arenberg, D. A., Reddy, R. C., Akulapalli, S., Thannickal, V. J., Standford, T. J., Andrews, P. C., and Ommen, G. S. (2006) Differential protein expression profiling by ITRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. J. Proteome Res. 5, 1143–1154

41. Tang, H. Y., Ali-Khan, N., Echan, L. A., Levenskova, N., Rux, J. J., and Speicher, D. W. (2005) A novel four-dimensional strategy combining protein and peptide separation methods enables detection of low-abundance proteins in human plasma and serum proteomes. Proteomics 5, 3329–3342