Quantitative Cancer Proteomics: Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) as a Tool for Prostate Cancer Research*

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Microarrays have been the primary means for large-scale analyses of genes implicated in cancer progression. However, more recently a need has been recognized for investigating cancer development directly at the protein level. In this report, we have applied a comparative proteomic technique to the study of metastatic prostate cancer. This technology, termed stable isotope labeling with amino acids in cell culture (SILAC), has recently gained popularity for its ability to compare the expression levels of hundreds of proteins in a single experiment. SILAC makes use of ^{12}C- and ^{13}C-labeled amino acids added to the growth media of separately cultured cell lines, giving rise to cells containing either "light" or "heavy" proteins, respectively. Upon mixing lysates collected from these cells, proteins can be identified by tandem mass spectrometry. The incorporation of stable isotopes also allows for a quantitative comparison between the two samples. Using this method, we compared the expression levels for more than 440 proteins in the microsomal fractions of prostate cancer cells with varying metastatic potential. Of these, 60 were found elevated greater than 3-fold in the highly metastatic cells, whereas 22 were reduced by equivalent amounts. Western blotting provided further confirmation of the mass spectrometry-based quantification. Our results demonstrate the applicability of this novel approach toward the study of cancer progression using defined cell lines. ***Molecular & Cellular Proteomics 3:729–735, 2004.***

Prostate cancer is currently the most common malignancy diagnosed in men and is estimated to account for over 230,000 new cancer diagnoses in the United States in 2004 (1). Changes in gene expression that occur during the development of prostate cancer have been extensively studied using DNA microarrays (2, 3). This genomic method offers the ability to compare the expression levels of thousands of genes in a single experiment. In many cases, however, gene expression levels do not accurately predict protein levels because other control mechanisms exist, including post-transcriptional regulatory mechanisms (4, 5). Protein expression can only be determined by direct measurement of protein levels. To date, analyses of protein levels in cancer have been performed primarily using two-dimensional (2D)[1] PAGE or surface-enhanced laser desorption/ionization (SELDI) mass spectrometry (6, 7). SELDI in particular has been used to demonstrate altered protein expression patterns in cancer but does not provide easy identification of individual proteins (8).

Stable isotope labeling with amino acids in cell culture, or SILAC, has recently emerged as a valuable proteomic technique (9–13). Using SILAC, cells representing two biological conditions can be cultured in amino acid-deficient growth media supplemented with ^{12}C- or ^{13}C-labeled amino acids. The proteins in these two cell populations effectively become isotopically labeled as "light" or "heavy." Upon isolation of proteins from these cells, samples can then be mixed in equal ratios and processed using conventional techniques for tandem mass spectrometry. Given that corresponding light and heavy peptides from the same protein will co-elute during chromatographic separation into the mass spectrometer, relative quantitative information can be gathered for each protein by calculating the ratio of intensities of the two peaks produced in the peptide mass spectrum (MS scan). Furthermore, sequence data can be acquired for these peptides by fragment analysis in the product ion mass spectrum (MS/MS scan) and used for accurate protein identification. Finally, when more than one peptide is identified from the same protein, the quantification is redundant, providing increased confidence in both the identification and quantification of the protein.

To identify proteins that may play a role in cancer progression, we chose to evaluate prostate carcinoma cell lines

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[1] The abbreviations used are: 2D, two-dimensional; SILAC, stable isotope labeling with amino acids in cell culture; SELDI, surface-enhanced laser desorption/ionization; PBS-T, phosphate-buffered saline-Tween 20; EGF, epidermal growth factor; MS, peptide mass spectrum; MS/MS, product ion mass spectrum.

PC3M and PC3M-LN4. Both of these cell types originated from the human prostate cancer PC3 line and were found to exhibit low (PC3M) and high (PC3M-LN4) metastatic potential, respectively (14). Since PC3M and PC3M-LN4 cells ultimately arose from the same cell line, we hypothesize that major changes observed in protein abundance may reflect their differences in metastatic potential. To show the feasibility of using the SILAC method to identify protein changes that may correlate with the metastatic phenotype, we applied this technique to the analysis of proteins in the PC3M and PC3M-LN4 cell lines. The identification of specific protein changes serves as a starting point for further studies, which will forward our understanding of the molecular mechanisms underlying metastasis and provide potential prognostic markers for prostate cancer.

EXPERIMENTAL PROCEDURES

Cell Culture and Protein Preparation—PC3M and PC3M-LN4 cells were grown at 37 °C in L-lysine-depleted RPMI 1640 media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% dialyzed fetal bovine serum (Invitrogen Corporation, Carlsbad, CA), antibiotics, and either $^{12}C_6$- (PC3M) or $^{13}C_6$- (PC3M-LN4) L-lysine (98% purity; Cambridge Isotope Laboratories, Andover, MA). Cells were plated at 2.0×10^5 cells/15-cm culture dish into six dishes each, and culture media was replaced every 2 days until 60–80% confluency was reached. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), and lysis and preparation of microsomal fractions were carried out according to Han *et al.* (15). Microsomal pellets were dissolved by boiling in 50 mM Tris-HCl, pH 8.3, 5 mM EDTA, and 2% SDS. Relative protein concentrations were measured using the bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL).

Protein Separation and In-gel Digestion—Equal amounts of protein from each sample were mixed at a 1:1 ratio, boiled in SDS-PAGE sample buffer, separated on a 10% Tris-glycine gel, and lightly stained using Coomassie Blue. The gel lane was excised and cut horizontally into 13 sections of similar size. Excised sections were cut into ~1-mm^3 pieces and destained using 50% acetonitrile/50% 50 mM ammonium bicarbonate solution, followed by dehydration in 100% acetonitrile for 10 min. Acetonitrile was discarded, and gel pieces were placed under vacuum centrifugation until completely dry. Each sample was then incubated overnight in a 10 ng/μl trypsin solution (in 50 mM ammonium bicarbonate). Peptides were extracted with 5% formic acid/50% acetonitrile into PCR tubes, dried using vacuum centrifugation, and stored at −20 °C until analysis by mass spectrometry.

Protein Identification and Quantification—Microcapillary columns (0.1×100 mm) were packed in-house with Magic C18AQ reversed-phase resin (5 μm) (Michrom BioResources, Inc., Auburn, CA). Samples were directly loaded onto the column using a FAMOS autosampler (LC Packings, Sunnyvale, CA) and an Agilent 1100 high-performance liquid chromatography binary pump. A gradient of acetonitrile in 0.4% acetic acid and 0.005% heptafluorobutyric acid was developed at 200 nl/min by flow-splitting (16). The eluent was introduced directly to a QSTAR Pulsar mass spectrometer (MDS Sciex, Toronto, Canada) via electrospray ionization. Eluting peptides were selected for fragmentation in an automated fashion, and the Analyst software package was used to generate and submit the MS/MS spectra as a batch file to the Mascot search algorithm (Matrix Science, London, United Kingdom) using the human database from NCBI. Labeled proteins were identified by allowing a mass increase of 6 Da on lysine. Identified proteins were quantified by tracking pairs of labeled and nonlabeled peptides in the ion chromatogram using SILAC-specific software (MSQuant) developed and provided by M. Mann and colleagues at the University of Southern Denmark (Odense, Denmark). Protein abundances were calculated as ratios of the areas of the monoisotopic peaks of the labeled *versus* the nonlabeled peptides. Proteins identified only by peptides with Mascot scores below 40 were excluded from analysis and not included in the list of quantified proteins.

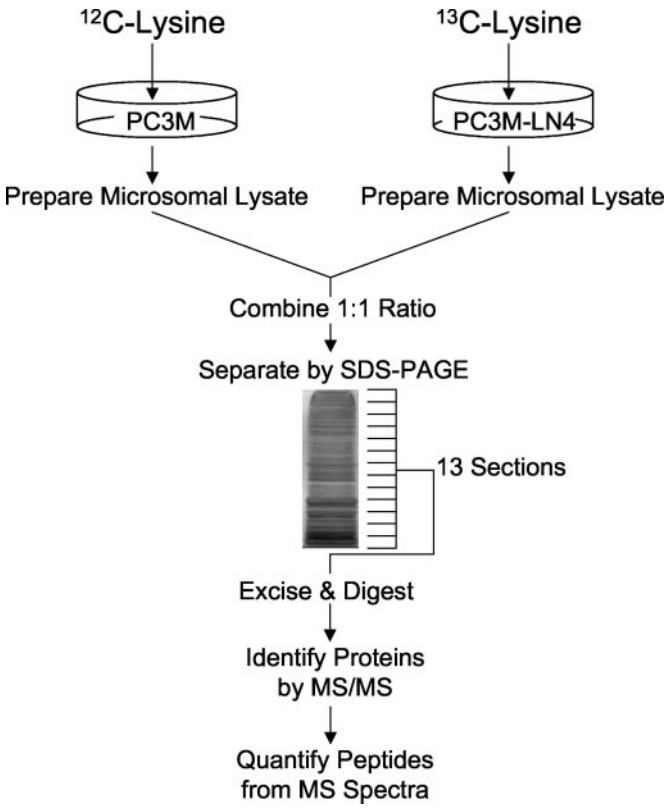

FIG. 1. **Schematic showing the SILAC method.** PC3M and PC3M-LN4 cells were cultured in media containing $^{12}C_6$- and $^{13}C_6$-lysine, respectively. Following isolation of microsomal proteins, equal amounts of protein from each sample were combined, creating a single sample that was then separated by SDS-PAGE. The entire gel lane was divided into 13 regions, and each section was processed for mass spectrometry as described in "Experimental Procedures." Quantitative data was determined from the ratio of peptide intensities produced from the light and heavy peptides in the MS spectra.

Western Blotting—Microsomal proteins were collected from PC3M and PC3M-LN4 as described. Following SDS-PAGE separation of equal amounts of protein from each sample on a 10% Tris-glycine gel, protein was transferred overnight at 4 °C onto nitrocellulose membrane (Schleicher & Schuell Bioscience, Inc., Keene, NH). After blocking for 1 h in 2% milk, membranes were washed in PBS-Tween 20 (PBS-T) and incubated with the following primary antibodies: anti-talin, anti-α-actinin 1, or anti-rab1B (1:200; Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Membranes were then washed three times in PBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Following three to five rinses in PBS-T, membranes were treated with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA) and protein intensities visualized using Hyperfilm ECL (Amersham Biosciences UK Ltd., Buckinghamshire, United Kingdom).

TABLE I
Twenty-five most highly upregulated microsomal proteins between low (PC3M) and high (PC3M-LN4) metastatic prostate cancer cells, as determined by SILAC

Protein name	GenBank acc. no.[a]	SILAC ratio[b]
Polyadenylate-binding protein 1	3183544	41.78 ± 11.68 (3)
Talin	4235275	16.54 (1)
Chaperonin (HSP60)	306890	13.85 ± 1.56 (4)
Laminin-binding protein	34234	13.16 ± 1.82 (2)
Eukaryotic translation initiation factor 3, subunit 10 θ, 150/170 kDa	4503509	9.71 ± 2.69 (4)
Vimentin	340219	9.55 (1)
Developmentally regulated GTP-binding protein 1	4758796	9.48 (1)
Annexin I	4502101	8.28 ± 1.31 (5)
Malate dehydrogenase, mitochondrial precursor	6648067	7.93 ± 2.17 (2)
B-cell receptor-associated protein	1673514	7.23 (1)
Proliferation-associated 2G4, 38 kDa	5453842	7.22 ± 1.21 (7)
Translation initiation factor eIF-2 β chain	87941	6.58 ± 1.37 (4)
Annexin XI	4557317	6.56 ± 0.65 (3)
Citrate synthase precursor	4758076	6.27 ± 1.24 (2)
ATP synthase, H^+-transporting, mitochondrial F1 complex, β polypeptide	32189394	5.92 (1)
Voltage-dependent anion-selective channel protein 1	10720216	5.75 ± 0.83 (2)
Unnamed protein product	22760762	5.71 ± 0.59 (5)
Peroxiredoxin 3 isoform a precursor	5802974	5.69 (1)
Phenylalanine-tRNA synthetase-like protein	4758340	5.67 ± 1.45 (2)
Phenylalanyl-tRNA synthetase β chain	12643818	5.43 ± 0.69 (4)
NSAP1 protein	5031512	5.43 ± 0.11 (2)
Lysyl-tRNA synthetase	11095909	5.20 ± 0.66 (2)
Annexin VII isoform 1	4502111	5.13 (1)
DNA-binding protein	181914	4.94 ± 1.04 (2)
Translation elongation factor EF-Tu-like protein P43 precursor, mitochondrial	7443384	4.81 (1)

[a] GenBank accession number.
[b] SILAC ratio refers to fold increase in protein expression, as described in "Experimental Procedures," with number of lysine-containing peptides quantified from each protein shown in parentheses.

RESULTS AND DISCUSSION

The SILAC strategy has previously been applied to a single cell line under two variable conditions (9, 11–13). Mann and colleagues used this approach in HeLa cells to examine changes associated with epidermal growth factor (EGF) signaling (11). Coupled with affinity purification to assess specific protein-protein interactions, their studies resulted in the identification and relative quantification of 228 proteins, with 28 of those showing a significant enrichment upon stimulation by EGF.

This is the first study utilizing the SILAC technique to examine two independent cell lines. In different cell lines, protein expression levels will be vastly different reflecting their unique cell type. To minimize these differences, we chose sub-lines arising from the same cell and expect similar patterns of expression among many of their proteins. We used SILAC analysis to examine the relative abundances of specific proteins in prostate cancer cells exhibiting low (PC3M) and high (PC3M-LN4) metastatic potential. Fig. 1 provides a schematic diagram of the SILAC procedure.

Because the levels of many membrane proteins may have an effect on cell motility, and therefore metastasis, identifying these changes in cell lines of differing metastatic potential could aid in our understanding of the underlying mechanisms of this process. Clearly, membrane and membrane-associated proteins play key roles cell adhesion and migration. Focal adhesion proteins such as focal adhesion kinase have been shown to be involved in cell motility (17, 18), and many other proteins localized to focal adhesions are important in transmitting signals that mediate differential adhesion and migration (19). To enrich for membrane and membrane-associated proteins, we isolated microsomal fractions (15) from PC3M and PC3M-LN4 cells (14), which were grown under light and heavy isotopic conditions, respectively. Our findings resulted in the identification of nearly 1,000 proteins. From these, relative quantitative information was gathered for 444 proteins (Tables I and II and supplemental data) because these were identified by lysine-containing tryptic peptides. Of the 444 proteins quantified, 60 showed greater than 3-fold up-regulation in the highly metastatic PC3M-LN4 line, whereas 22 proteins were downregulated by similar amounts. Fig. 2 shows the SILAC ratio distribution for all 444 proteins quantified.

We note that nearly one-third of the proteins in this study were quantified by a single lysine-containing peptide. While the vast majority of the proteins were identified by additional arginine-containing peptides, only two (thioredoxin (GenBank accession no. 4507745) and coatomer protein complex, subunit γ 1 (GenBank accession no. 11559929), supplemental data) out of our list of 444 proteins were identified and quan-

TABLE II
Twenty-five most highly downregulated microsomal proteins between low (PC3M) and high (PC3M-LN4) metastatic prostate cancer cells, as determined by SILAC

Protein name	GenBank acc. no.[a]	SILAC ratio[b]
Predicted osteoblast protein	7661714	0.35 (1)
Adenylyl cyclase-associated protein	5453595	0.34 ± 0.06 (6)
Chain A, crystal structure of human phosphoglucose isomerase	14488680	0.34 ± 0.04 (5)
Heat shock 27 kDa protein 1	4504517	0.33 ± 0.06 (3)
Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	5453880	0.30 ± 0.05 (3)
DEAD-box protein 3 (helicase-like protein 2) (HLP2) (DEAD-box, X isoform)	3023628	0.29 (1)
Membrane component, chromosome 11, surface marker 1	20127488	0.29 (1)
Template activating factor-I, splice form α	2136258	0.29 ± 0.03 (4)
Ubiquitin (high molecular mass conjugates)	136670	0.29 (1)
α-Actinin 1 (α-actinin cytoskeletal isoform)	112959	0.25 ± 0.05 (12)
Annexin III	4826643	0.25 ± 0.05 (4)
Protein kinase, cAMP-dependent, catalytic, α	4506055	0.24 (1)
Oxidized protein hydrolase	7144648	0.23 (1)
BRCA2 and CDKN1A-interacting protein isoform BCCIPα	7706581	0.22 ± 0.04 (3)
Transglutaminase 2	4759228	0.22 (1)
Protein kinase, cAMP-dependent, regulatory, type I, α	4506063	0.21 ± 0.05 (3)
Nucleobindin 1	20070228	0.18 (1)
Nonhistone chromosomal protein HMG-1	478813	0.17 ± 0.09 (8)
Synaptojanin 2A	25361067	0.15 (1)
Aldehyde dehydrogenase 1A3	4502041	0.14 ± 0.04 (7)
Chain A, crystal structure of the human co-chaperone P23	9257073	0.14 ± 0.09 (2)
Similar to 60S ribosomal protein L14	20555164	0.13 (1)
Lipocalin 1 precursor	4504963	0.11 ± 0.01 (2)
Aminopeptidase N (AA 1 - 967)	28678	0.06 ± 0.01 (2)
Eukaryotic translation initiation factor 2B, subunit 2 β, 39 kDa	7657058	0.05 (1)

[a] GenBank accession number.
[b] SILAC ratio refers to fold increase in protein expression, as described in "Experimental Procedures," with number of lysine-containing peptides quantified from each protein shown in parentheses.

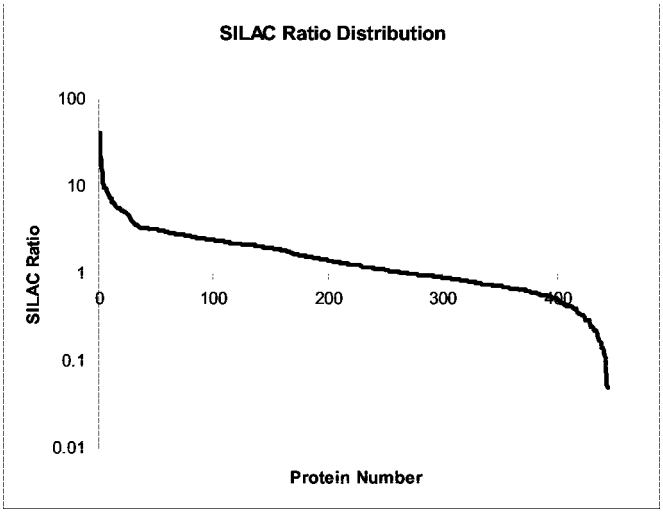

FIG. 2. **Distribution of protein expression ratios as determined by SILAC.** The SILAC ratio for each protein (n = 444) represents the relative expression difference between high (PC3M-LN4) and low (PC3M) metastatic prostate cancer cells. Proteins were sorted and plotted by SILAC ratio.

tified by a single peptide. However, each of the peptides used to identify and quantify these two proteins had Mascot scores greater than 60, providing high confidence in both their identification and quantification. Furthermore, the MS/MS spectra for all peptides used for quantification in this report were manually inspected and verified as correct answers.

To maximize the number of proteins identified and quantified, the complex mixture of proteins in the SDS-PAGE gel lane was excised and divided into 13 sections corresponding to different molecular masses, each serving as a separate sample that was then subjected to trypsin digestion followed by MS and MS/MS analysis. Increasing the number of sections enhanced our ability to identify and quantify low-abundance proteins that may have otherwise been masked by proteins expressed at very high levels. This occasionally resulted in duplications within our initial list of proteins. These duplicated proteins showed comparable SILAC ratios, so duplicate values for single proteins were therefore omitted from our final list. However, ubiquitin was one exception that did not exhibit similar ratios. Ubiquitin was found in sections corresponding to multiple molecular masses, which can be expected due to its covalent attachment to proteins of varied sizes. Since mass-specific differences in the levels of ubiquitination may be a result of differing metastatic phenotypes, duplications of ubiquitin were retained in our final list of quantified proteins (supplemental data).

Fig. 3 illustrates the coupling of stable isotopes and mass spectrometry for both the identification of proteins and the quantification of their relative expression levels. Following

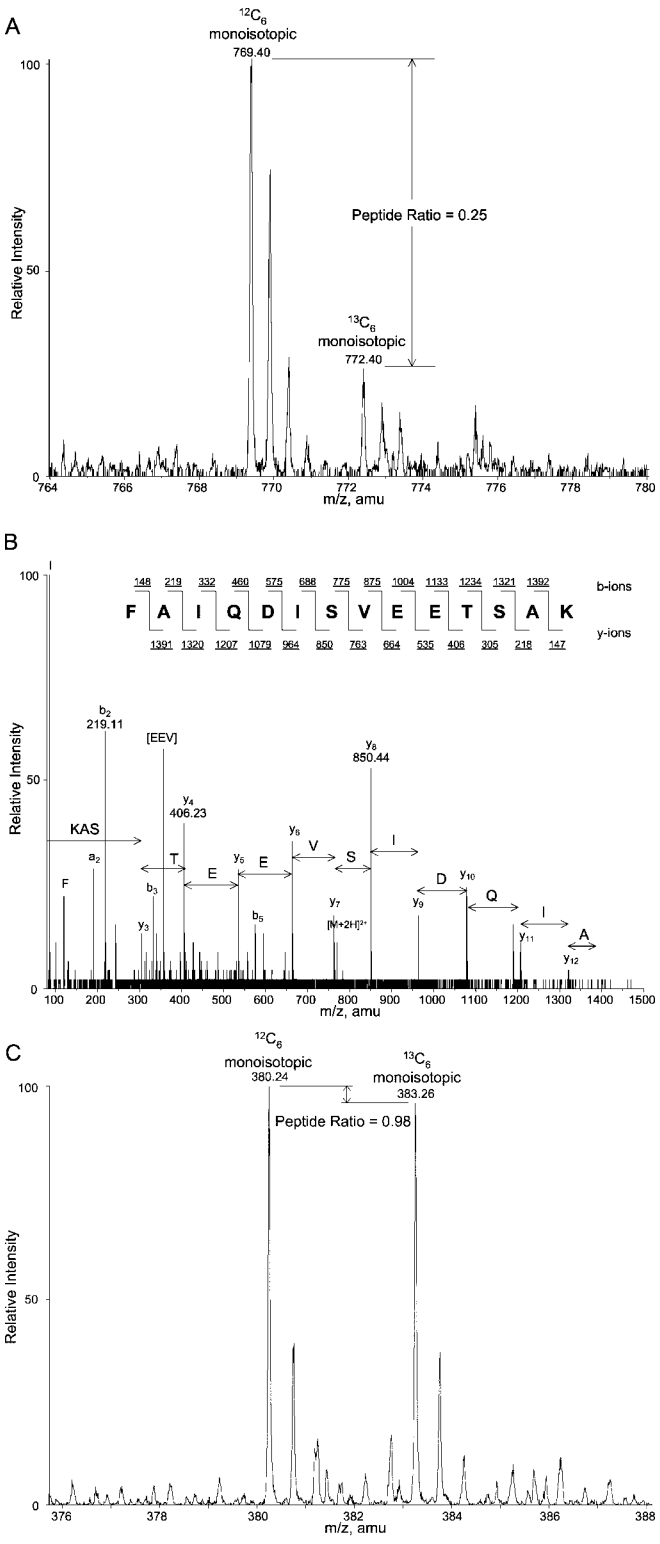

FIG. 3. **Identification and quantification of SILAC peptides.** *A*, MS spectrum showing a SILAC peptide pair from α-actinin 1. Ratio was determined by calculating the areas under the monoisotopic peaks from the nonlabeled and labeled peptides. The peptide pair shown has a charge state of 2^+, giving rise to an *m/z* difference of 3 mass units. Additional peaks of decreasing intensity following each monoisotopic peak correspond to naturally occurring isotope peaks. *B*, tandem mass (MS/MS) spectrum of α-actinin 1 peptide shown in *A*. Masses of expected b- and y-type fragment ions are shown with identified peptide. Peaks denoted by I and F represent immonium ions formed during collision-induced dissociation from isoleucine and phenylalanine, respectively. *C*, control SILAC peptide (IISLDAK) pair from glutamyl-prolyl tRNA synthetase (GenBank accession no. 4758294) showing ratio of 0.98. For this control experiment, PC3M cells were cultured under both ^{12}C and ^{13}C conditions, mixed at a 1:1 ratio based on protein concentration, and identification and quantification was carried out as described in "Experimental Procedures." Glutamyl-prolyl tRNA synthetase exhibited a greater than 2-fold increase in expression (SILAC ratio 2.27; supplemental data) between the PC3M and PC3M-LN4 lines.

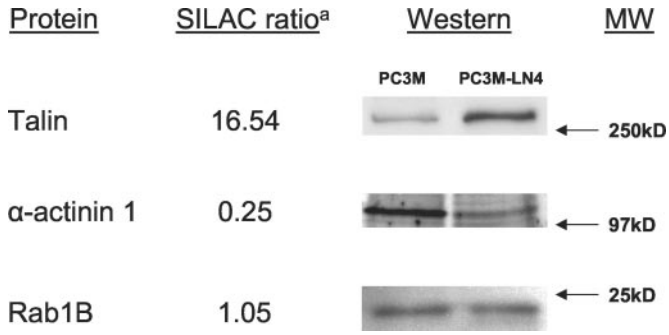

FIG. 4. **Immunoblots of selected proteins from microsomal lysates of PC3M and PC3M-LN4 cell lines.** Antibodies to talin (270 kDa), α-actinin 1 (103 kDa), and rab1B (22 kDa) were used to demonstrate agreement between protein expression differences determined by SILAC and immunoblotting. [a] SILAC ratio refers to fold increase in protein expression, as described in "Experimental Procedures."

in-gel trypsin digestion of an isotopically mixed protein sample resolved by SDS-PAGE, peptides were eluted based on hydrophobic properties from microcapillary columns and ionized into the mass spectrometer using electrospray ionization. Because the difference in hydrophobicity between peptides containing $^{12}C_6$- and $^{13}C_6$-lysine is insignificant, peptides with the same amino acid sequence will co-elute, regardless of any ^{12}C or ^{13}C modification. Relative quantification is performed from single peptides in the MS mode, relating the two monoisotopic peaks from peptides containing $^{12}C_6$- and $^{13}C_6$-lysine (Fig. 3*A*). The protein is then identified from the MS/MS fragmentation spectra (Fig. 3*B*) using the Mascot algorithm. To ensure that isotopically unmodified and modified peptides possess the same ionization efficiency, PC3M cells were cultured under both light and heavy conditions and lysates were mixed at a 1:1 ratio. Analyses of the monoisotopic peaks in the MS spectra showed an expected 1:1 ratio. An example is illustrated in Fig. 3*C*.

To further confirm our SILAC results, three proteins for which antibodies were readily available were examined by Western blotting. SILAC ratios of 16.54 and 0.25 were observed for talin and α-actinin 1, respectively (see Tables I and II). Immunoblots using separate microsomal collections were

employed to verify these findings (Fig. 4). The level of rab1B, a small GTPase involved in protein transport (20), was used as a loading control because relatively no change was observed by SILAC (supplemental data). Using densitometry to normalize for differences in background, 1:1 expression levels of rab1B Western blot bands were verified using TotalLab 2.00 (Nonlinear Dynamics, Ltd., Newcastle, United Kingdom).

There are a number of recent reports on differential gene expression in prostate cancer. One proteomic study found at least a 2-fold up-regulation of vimentin, ATP synthase, and α-tubulin in prostate cancer cells exhibiting high tumorigenicity as compared with poorly tumorigenic cells (21). Our results suggest that these proteins may also be implicated in metastasis, showing SILAC ratios of 9.55, 5.92, and 2.17, respectively (Table I and supplemental data).

Further analysis of the highly up- and downregulated proteins in the metastatic PC3M-LN4 cells (Tables I and II) reveals that many are involved in translation regulation. For example, the expression levels of polyadenylate-binding protein 1, an RNA-binding protein with important roles in translation initiation and mRNA stabilization (22), show an increase of greater than 40-fold in the highly metastatic cells (Table I). A study put forth by Jechlinger *et al.* uncovered a number of differentially regulated genes involved in tumor progression, with specific roles in cell migration, local invasion, and metastasis (23). Interestingly, they concluded that ~15% of all the regulated genes were translationally controlled. Taken together, these results further highlight the importance of translational control in cancer.

Large-scale analyses of metastatic prostate cancer have been performed using microarrays and have resulted in the discovery of new patterns of gene expression (2, 24). However, there are discrepancies in the regulated expression levels between mRNA and protein in prostate cancer, as previously suggested (5). A major advantage of using quantitative mass spectrometry not afforded by microarrays is that it allows for cellular analysis directly at the level of protein expression. Consequently, increasing reports on quantifying protein expression in cancer have resulted from rapid advances in proteomic strategies and mass spectrometry. Most of these studies have focused efforts using 2D-PAGE (25), providing a relatively small amount of data on protein identification and quantification due to the low throughput and poor reproducibility of coupling 2D-PAGE with mass spectrometry. Using the SILAC strategy, we report the largest study to date on protein identification and relative quantification in a model of metastatic prostate cancer. This study provides a proof of principle and leads the way for further experiments in additional models of metastasis.

In addition, a number of proteins were identified with altered membrane expression levels that had not previously been found to be associated with metastasic cells. Among those with the most variation in expression is an uncharacterized protein (GenBank accession no. 22760762; Table I) showing over 5-fold up-regulation in the highly metastatic PC3M-LN4 line. This gene product also contains a predicted mRNA-binding domain and may therefore play a role in translation. Further studies are being directed toward determining if this protein has a direct influence on prostate cancer metastasis.

This dataset of 444 quantified proteins (from the identification of more than 1,000) represents the largest SILAC experiment to date. This number could be further extended by either i) increasing the gel lane fractionation from 13 to 20 or more regions or ii) utilizing both ^{13}C-containing arginine and lysine during metabolic incorporation.

In conclusion, we have successfully shown that this technology can be applied to closely related cells and also demonstrated the direct application of this method to a model of metastatic prostate cancer. Future experiments will involve the use of additional cancer cell lines to narrow the search for predictive biomarkers. While we have shown that SILAC can be applied to a model of metastasis, we suggest that variations in cell migration, invasion, drug resistance, and metabolism are just a few of the many cellular properties for which SILAC could prove useful for quantitative proteomic analyses.

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S The on-line version of this manuscript (available at http://www.mcponline.org) contains supplemental material.

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