THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 9, Issue of February 28, pp. 7607–7616, 2003
Printed in U.S.A.

Global Profiling of the Cell Surface Proteome of Cancer Cells Uncovers an Abundance of Proteins with Chaperone Function*

Received for publication, October 11, 2002, and in revised form, December 13, 2002
Published, JBC Papers in Press, December 18, 2002, DOI 10.1074/jbc.M210455200

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There is currently limited data available pertaining to the global characterization of the cell surface proteome. We have implemented a strategy for the comprehensive profiling and identification of surface membrane proteins. This strategy has been applied to cancer cells, including the SH-SY5Y neuroblastoma, the A549 lung adenocarcinoma, the LoVo colon adenocarcinoma, and the Sup-B15 acute lymphoblastic leukemia (B cell) cell lines and ovarian tumor cells. Surface membrane proteins of viable, intact cells were subjected to biotinylation then affinity-captured and purified on monomeric avidin columns. The biotinylated proteins were eluted from the monomeric avidin columns as intact proteins and were subsequently separated by two-dimensional PAGE, transferred to polyvinylidene difluoride membranes, and visualized by hybridization with streptavidin-horseradish peroxidase. Highly reproducible, but distinct, two-dimensional patterns consisting of several hundred biotinylated proteins were obtained for the different cell populations analyzed. Identification of a subset of biotinylated proteins among the different cell populations analyzed using matrix-assisted laser desorption ionization and tandem mass spectrometry uncovered proteins with a restricted expression pattern in some cell line(s), such as CD87 and the activin receptor type IIB. We also identified more widely expressed proteins, such as CD98, and a sushi repeat-containing protein, a member of the selectin family. Remarkably, a set of proteins identified as chaperone proteins were found to be highly abundant on the cell surface, including GRP78, GRP75, HSP70, HSP60, HSP54, HSP27, and protein disulfide isomerase. Comprehensive profiling of the cell surface proteome provides an effective approach for the identification of commonly occurring proteins as well as proteins with restricted expression patterns in this compartment.

The surface membrane is a cellular compartment of substantial interest. Comprehensive profiling of proteins expressed on the cell surface could provide a better understanding of the manner in which the cell surface proteome is regulated and how it responds to a variety of intracellular and extracellular signals. This compartment is also rich in therapeutic targets. For example, the discoveries that the gene for a growth factor receptor (*HER*2) is amplified in breast tumors and its protein product is overexpressed at the cell surface have led to an effective form of therapy for breast cancer utilizing an antibody that targets HER2 (1). Also, elucidation of the role of growth factor receptors expressed on the cell surface in signaling and in uncontrolled cell proliferation, as in the case of epidermal growth factor receptor, has led to the development of new anticancer therapies that target specific components of the epidermal growth factor receptor signal transduction pathway. Selective compounds have been developed that target the extracellular ligand binding region of epidermal growth factor receptor (2). Thus, the development of an effective strategy for the comprehensive analysis of surface membrane proteins would have important implications. In cancer, cell surface proteins that are restricted in their expression to specific cancer(s) or that undergo restricted modifications could be utilized for antibody-based therapy, as in the case of HER2 or for vaccine development or other forms of immunotherapy. Signaling pathways regulated by surface membrane proteins or receptors could also be targeted for a drug-based therapy.

There is currently a paucity of data pertaining to the comprehensive analysis of surface membrane proteins due, in part, to a lack of effective strategies to profile the proteome of surface membranes. Problems associated with the profiling of this compartment stem from the limited abundance of surface membrane proteins and the difficulty in resolving and identifying them. Protein tagging technologies have been available for a long time and have been utilized in a variety of applications, yet surprisingly, very few studies have attempted to incorporate protein tagging as part of strategies to enhance sensitivity of procedures for quantitative protein analysis, such as two-dimensional gels. For example, protein radio-iodination has been utilized for many years in different types of protein studies, yet few publications have emerged that were based on the analysis of radioiodinated proteins in complex mixtures, when compared with the vast literature that exists for protein analysis and detection by silver staining. Novel approaches to improve the detection of proteins by post-harvest alkylation and subsequent radioactive labeling with either [^{3}H]iodoacetamide or ^{125}I have been described and are promising (3).

The high affinity and specificity of avidin-biotin interactions have been exploited for diverse applications in immunology, histochemistry, *in situ* hybridization, affinity chromatography, and many other areas (4–8). Biotinylation reagents provide the "tag" that transforms poorly detectable molecules into probes, which can be recognized by a labeled detection reagent. Once tagged with biotin, a molecule of interest such as an antibody or receptor ligand can be used to probe cells, tissues, or proteins

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immobilized on blots or arrays. The tagged molecule is detected with a labeled avidin conjugate. Although the binding of biotin to native avidin or streptavidin is essentially irreversible, modified avidins can bind biotinylated probes reversibly, making them valuable reagents for isolation and purification of biotinylated molecules from complex mixtures. We have implemented a biotinylation-based strategy for targeting surface membrane-derived proteins, to allow capture of these proteins, thereby providing substantial enrichment, and increased sensitivity through the use of avidin labels for detection. There have been prior studies that have combined protein biotinylation with two-dimensional gels (9). In these studies, protein identification was largely based on the use of antibodies to specific proteins. However, very recently, a global surface protein biotinylation strategy, coupled with the use of mass spectrometry, was applied to *Helicobacter pylori*, leading to the identification of 18 proteins (10). We here report on the global profiling of proteins on the surface of a variety of cancer cell types, which has uncovered an abundance of proteins with chaperone function.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents, including RPMI 1640 (containing L-glutamine) and Dulbecco's modified Eagle's medium (DMEM,[1] containing L-glutamine, sodium pyruvate, and pyridoxine hydrochloride), Dulbecco's phosphate-buffered saline (D-PBS), fetal calf serum, and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). The ImmunoPure Immobilized Monomeric Avidin and EZ-Link sulfo-NHS-LC-biotin were obtained from Pierce (Rockford, IL). D-Biotin was obtained from US Biologicals (Cleveland, OH). The two-well chamber slides were from Nalge-Nunc (Napierville, IL). The rabbit anti-α EphB4 and the goat anti-GRP78-N antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-HSP70 antibody was purchased from Sigma (St. Louis, MO). Ficoll-Paque Plus, streptavidin-biotinylated horseradish peroxidase complex, and HRP-conjugated goat anti-rabbit IgG and the ECL (Enhanced Chemiluminescence) kits were obtained from Amersham Biosciences (Arlington Heights, IL). The biotinylated anti-streptavidin IgG was obtained from Vector Laboratories (Burlingame, CA). Streptavidin R-phycoerythrin, Alexa 488 highly cross-adsorbed goat anti-rabbit IgG, and Alexa 488 donkey anti-goat IgG were obtained from Molecular Probes (Eugene, OR). Immobilon-P PVDF (polyvinylidene difluoride) membranes were purchased from Millipore Corp. (Bedford, MA). Acrylamide used in the one-dimensional electrophoresis, urea, ammonium persulfate, and piperazine diacrylamide were all purchased from Bio-Rad (Rockville Centre, NY). Acrylamide used in the two-dimensional electrophoresis was purchased from Serva (Crescent Chemical, Hauppauge, NY), and carrier ampholytes (both pH 4–8 and pH 3.5–10) and Nonidet P-40 were purchased from Gallard/Schlessinger (Carle Place, NY). All other reagents and chemicals were obtained from either Fisher or Sigma and were of the highest purity available.

Cell Culture—Established human cancer cell lines cultured as adherent monolayers (SH-SY5Y neuroblastoma cells, A549 lung adenocarcinoma cells, and LoVo colon adenocarcinoma cells) were propagated at 37 °C in a 6% CO_2-humidified incubator in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The cells were passaged weekly upon reaching confluence. The non-adherent (Sup-B15 acute lymphoblastic leukemia-B cell) human Leukemia cell line was grown at 37 °C in a 6% CO_2-humidified incubator in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The cells were passaged weekly. Freshly isolated ovarian tumor cells were obtained from ascites fluid by centrifugation (1000 × *g*, 10 min at room temp), followed by washing with DMEM (without added serum or protein), then purified on a 3-ml layer of Ficoll-Paque (400 × *g* for 40 min at 20 °C). The tumor cell-containing band was harvested from the Ficoll-Paque and washed three times in D-PBS (without added serum or protein).

Biotinylation of Membrane Proteins—Adherent monolayers of cultured cells (SH-SY5Y, A549, and LoVo) grown in 75-cm^2 tissue culture dishes were washed three times with D-PBS (without added serum or protein). 10 ml of DMEM (without added serum or protein) containing 0.5 mg/ml EZ-Link sulfo-NHS-LC-biotin was added, and the cells were incubated at 37 °C for 10 min. The biotinylation reaction was terminated by addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM. Following biotinylation, the cells were washed in PBS and harvested by scraping the cell monolayers in PBS containing 2% Nonidet P-40. The cells were further disrupted by brief sonication. Sup-B15 acute lymphoblastic leukemia-B cells were harvested by centrifugation (1000 × *g*, 10 min at room temp) and washed three times with RPMI 1640 (without added serum or protein). Sup-B15 cells and ovarian tumor cells were suspended at 2.5×10^7 cells/ml in RPMI 1640 and DMEM, respectively (without added serum or protein), containing 0.5 mg/ml EZ-Link sulfo-NHS-LC-biotin and incubated at 37 °C for 10 min. The biotinylation reaction was terminated by addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM. Following biotinylation, the cells were washed in PBS, pelleted by centrifugation, and solubilized in PBS containing 2% Nonidet P-40. The cells were further disrupted by brief sonication.

Purification of Biotinylated Membrane Proteins—Solubilized biotinylated membrane proteins from the various cell populations were purified on ImmunoPure immobilized monomeric avidin columns (Pierce), with modifications of the protocol supplied by the manufacturer. Briefly, 2.5-ml columns of immobilized monomeric avidin were prepared and extensively washed with PBS. The columns were washed with 2 mM D-biotin in PBS to block any non-reversible biotin binding sites on the column. The loosely bound biotin was removed from the reversible biotin binding sites by washing with 12 ml of 0.1 M glycine (pH 2.8), and the columns were then extensively washed with PBS. The disrupted cells with membrane protein biotinylation were again subjected to sonication, after which the solubilization solution was clarified by centrifugation (14,000 rpm for 20 min at 4 °C). The solubilization solution was passed through the immobilized monomeric avidin columns three times, after which the column was again extensively washed with PBS containing 1% Nonidet P-40. The bound biotinylated proteins were eluted from the column with 5 mM D-biotin in PBS containing 1% Nonidet P-40. Fractions containing eluted protein were concentrated on Centricon YM-3 columns (Millipore).

Two-dimensional PAGE and Western Blotting—Biotinylated proteins in the tumor cells and in the cell lines were analyzed by two-dimensional PAGE (11). Briefly, proteins were solubilized with lysis buffer, containing 8 M urea, 2% pH 3.5–10 carrier ampholytes, 2% β-mercaptoethanol, 2% Nonidet P-40, and 10 mM phenylmethylsulfonyl fluoride. Isoelectric focusing was carried out using either pH 4–8 carrier ampholyte-based tube gels for 13,200 V-h at room temperature or using immobilized 4–10 pH gradient (IPG)-based strips (12). One-dimensional gels were loaded onto a cassette containing the second-dimensional gel, after equilibration in second-dimensional sample buffer (125 mM Tris (pH 6.8), containing 10% glycerol, 2% SDS, 1% dithiothreitol, and bromphenol blue). Separation in the second dimension was performed by electrophoresis in 7–14% polyacrylamide gradient SDS gels, and the samples were electrophoresed until the dye front reached the opposite end of the gel. Some gels were silver-stained and digitized for pattern analysis as previously described (13). For some other gels, the resolved proteins were transferred to an Immobilon-P PVDF membrane. Unstained membranes were prepared for hybridization by incubation with blocking buffer (consisting of Tris-buffered saline (TBS) containing 1.8% nonfat dry milk and 0.1% Tween 20) for 2 h, then washed and incubated with a horseradish peroxidase-conjugated biotin-streptavidin complex (at a 1:400 dilution) for 40 min at room temperature. The membranes were washed five times with TBS containing 0.1% Tween 20, once in TBS, briefly incubated in ECL, and exposed to XAR-5 x-ray film. Patterns visualized were directly compared with comparable gel silver-stain patterns.

In-gel Enzymatic Digestion and Mass Spectrometry—Additional two-dimensional gels containing proteins eluted from avidin columns were silver-stained by successive incubations in 0.02% sodium thiosulfate for 2 min, 0.1% silver nitrate for 40 min, and 0.014% formaldehyde plus 2% sodium carbonate. The proteins of interest were excised from the two-dimensional gels and destained for 5 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate as described previously (14). Following three washes with water, the gel pieces were dehydrated in 100% acetonitrile for 5 min and dried for 30 min in a vacuum centrifuge.

[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; NHS, *N*-hydroxysuccinimide; IPG, immobilized 4–10 pH gradient; MALDI-TOF, matrix-assisted laser desorption time of flight; ESI, electrospray ionization; Q-TOF MS/MS, quadrupole time of flight tandem mass spectrometry; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; SSPE, saline/sodium phosphate/EDTA; ER, endoplasmic reticulum; GRP, glucose-regulated protein; HSP, heat-shock protein; TLR, toll-like receptor; IL-1, interleukin-1; ABC, ATP-binding cassette.

Digestion was performed by addition of 100 ng of trypsin (Promega) in 200 mM ammonium bicarbonate. Following enzymatic digestion for 18 h at 37 °C, the peptides were extracted twice with 50 μl of 60% acetonitrile/1% trifluoroacetic acid. After removal of acetonitrile in a vacuum centrifuge, the peptides were concentrated by using pipette tips (C18, Millipore, Bedford, MA).

Peptide mixtures were analyzed using either a PerSeptive Biosystems (Framingham, MA) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager-DE mass spectrometer, operated in delayed extraction mode, or by nanoflow capillary liquid chromatography coupled with electrospray quadrupole time of flight tandem mass spectrometry (ESI Q-TOF MS/MS) in the Q-TOF micro (MicroMass, Manchester, UK). The peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in acetonitrile containing 1% trifluoroacetic acid (0.5 μl of sample:0.5 μl of matrix). Peptides were selected in the mass range of 800–4000 Da. Spectra were calibrated using calibration mixture 2 of the Sequazyme peptide mass standards kit (PerSeptive Biosystems). MALDI-TOF MS gave a peptide mass fingerprint for each spot based on the molecular mass of trypsin-digested products. We compared the resulting masses with known trypsin digest protein sequence databases (SwissProt or NCBInr) using the MS-Fit data base search engine developed by the University of California at San Francisco (available at prospector.ucsf.edu/ucsfhtml3.2/msfit.htm). ESI MS/MS tandem spectra were recorded in the automated MS to MS/MS switching mode, with an *m/z*-dependent set of collision offset values. Singly to triply charged ions were selected and fragmented, with argon used as the collision gas. The acquired spectra were processed and searched against a non-redundant SwissProt protein sequence data base using the ProteinLynx global server (available at www.micromass.co.uk).

Immunofluorescence—The cell lines and tumor cells were grown in two-well chamber slides for 48 h and then fixed in 2% formaldehyde, freshly prepared from paraformaldehyde. The fixed cells were washed briefly in D-PBS. Aldehyde groups resulting from fixation were quenched in 50 mM L-lysine (in D-PBS), after which the fixed monolayers were washed three times in D-PBS (containing 1 mg/ml BSA). Each chamber was incubated with 0.5 ml of the appropriate primary antibody diluted as indicated in D-PBS (containing 2 mg/ml BSA) for 1 h at room temp. The cells were washed in D-PBS (containing 2 mg/ml BSA). After which they were incubated in 0.5 ml of D-PBS (containing 2 mg/ml BSA) containing either 10 μg/ml highly cross-adsorbed Alexa-488-conjugated goat anti-rabbit IgG, 20 μg/ml Alexa-488-conjugated donkey anti-goat IgG, or 20 μg/ml highly cross-adsorbed Alexa-488-conjugated goat anti-mouse IgG. The stained monolayers were washed three times in D-PBS (containing 1 mg/ml BSA) and three times in D-PBS, after which a glass coverslip was mounted on the monolayers in GEL/MOUNT (Biomeda Corp., Foster City, CA). Fluorescence images were visualized through a Zeiss 510LSM Confocal Laser Scanning microscope.

DNA Microarray Analysis—Total RNA was isolated using TRIzol reagent (Invitrogen), which was followed by clean-up on an RNeasy spin column (Qiagen), then the total RNA was used to generate cRNA probes. Preparation of cRNA, hybridization, and scanning of the HuGeneFL arrays were performed as previously described (65). Briefly, 5 μg of total RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice System, Invitrogen) with an oligo$(dT)_{24}$ primer containing a T7 RNA polymerase promoter site added 3′ of the poly-T (Genset, La Jolla, CA). Following second-strand synthesis, labeled cRNA was generated from the cDNA sample by an *in vitro* transcription reaction supplemented with biotin-11-CTP and biotin-16-UTP (Enzo, Farmingdale, NY). The labeled cRNA was purified on RNeasy spin columns (Qiagen). 15 μg of each cRNA was fragmented at 94 °C for 35 min in fragmentation buffer (40 mM Tris acetate (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate). 15 μg of fragmented cRNA was used to prepare 300 μl of hybridization mixture (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20) containing 0.1 mg/ml herring sperm DNA (Promega, Madison WI), 500 μg/ml acetylated BSA (Invitrogen), and control cRNAs for comparison of hybridization efficiency between arrays. Prior to hybridization, the mixtures were heated to 94 °C for 5 min, equilibrated at 45 °C for 5 min, and then clarified by centrifugation (16,000 × *g*) at room temperature for 5 min. Aliquots of each sample (10 μg of fragmented cRNA in 200 μl of hybridization mixture) were hybridized to HuGeneFL arrays at 45 °C for 16 h in a rotisserie oven set at 60 rpm. The arrays were then washed with non-stringent wash buffer (6× SSPE) at 25 °C, followed by stringent wash buffer (100 mM MES (pH 6.7), 0.1 M NaCl, 0.01% Tween 20) at 50 °C, and stained with streptavidin-R-phycoerythrin. The arrays were washed again with 6× SSPE, stained with biotinylated anti-streptavidin IgG, followed by a second staining with streptavidin-phycoerythrin, and a third washing with 6× SSPE. The arrays were scanned using the GeneArray scanner (Affymetrix). Data analysis was performed using GeneChip 4.0 software.

RESULTS

Profiling the Cell Surface Proteome by Protein Biotinylation—The approach we have implemented for the comprehensive profiling and identification of surface membrane proteins involves the selective biotinylation of the surface proteins of intact cells. Following separation by two-dimensional PAGE and transfer to PVDF membranes, the biotinylated proteins are visualized by hybridization with a streptavidin/horseradish peroxidase complex. Alternatively, biotinylated proteins are captured on an affinity column, followed by their separation and identification. We have applied this approach to the analysis of surface membranes of cancer cells and compared biotinylation patterns obtained for different cell lineages.

Fig. 1 displays the biotinylated proteins in whole cell lysates of A549 lung adenocarcinoma cells following cell surface labeling. It is evident that the pattern of visualized biotinylated proteins (Fig. 1*B*) is quite rich in separated proteins that are not visualized in silver-stained two-dimensional gels of the same whole cell lysates (Fig. 1*A*). Many of the resolved biotinylated proteins form trains of spots, as expected for proteins that undergo numerous post-translational modifications (*e.g.* glycosylation, phosphorylation, and sulfation). Although some proteins could be matched in their location between the silver-stained (Fig. 1*A*) and biotinylated (Fig. 1*B*) two-dimensional patterns, most biotinylated proteins did not have a match in the silver-stained two-dimensional pattern of whole cell lysates. This strongly suggests that we were able to obtain a selective and enhanced visualization of low abundance proteins by biotinylation of the surface membrane.

Capture and Purification of Biotinylated Surface Membrane Proteins—Because the population of surface membrane proteins that were biotinylated represented low abundance proteins at the whole cell level, it was necessary to utilize an enrichment procedure following biotinylation to allow identification of the biotinylated proteins. To this end, a column of immobilized monomeric avidin was utilized to bind biotinylated proteins, with their subsequent elution from the column in PBS containing 5 mM D-biotin, 1% Nonidet P-40. Column eluates from different cell populations were each concentrated ~400-fold, and aliquots were resolved by either two-dimensional PAGE or IPG, followed by silver staining for some aliquots or transfer to PVDF membranes for others. PVDF membranes were hybridized with streptavidin/horseradish peroxidase and visualized with the ECL reagents. Two-dimensional patterns for eluted proteins visualized by silver staining had remarkable similarity with two-dimensional patterns of proteins that were transferred to PVDF membranes, then hybridized with streptavidin/horseradish peroxidase and detected with the ECL reagent, indicating that most proteins captured on the column represented biotinylated proteins. Fig. 2 displays an IPG two-dimensional pattern for the A549 lung adenocarcinoma following column elution and silver staining. It shows similarity with the pattern for the same cell population obtained following a carrier ampolyte separation of a whole cell lysate of biotinylated A549 cells in the common pH separation region (Fig. 1). Moreover, patterns resolved by carrier ampolyte or IPG overlapped substantially for the same pH separation range (Figs. 1*B* and 2). Furthermore, Fig. 3 shows that the pattern of biotinylated proteins from ovarian tumor cells that were visualized by hybridization was highly similar to the pattern obtained from silver-stained gels of the same aliquot of eluted protein.

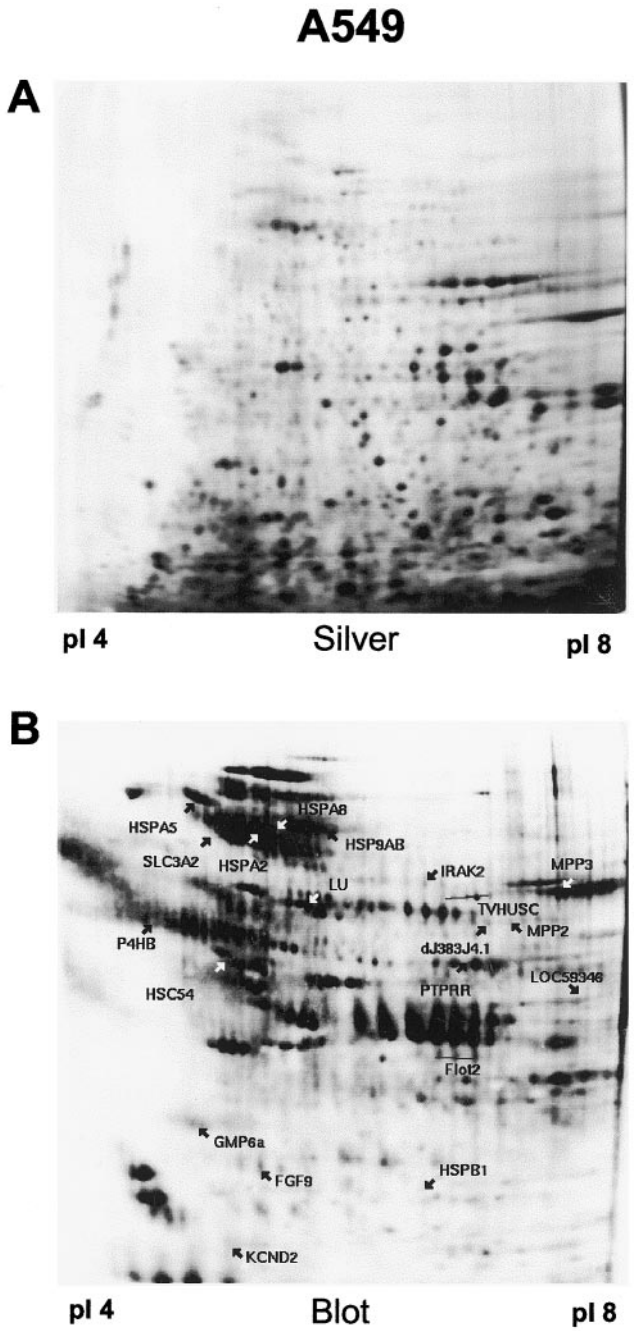

FIG. 1. **Visualization of surface biotinylation patterns in A549 lung adenocarcinoma cells.** *A*, two-dimensional PAGE analysis of cellular proteins from the A549 cell line. Solubilized proteins from A549 lung adenocarcinoma cells were resolved by two-dimensional PAGE using carrier ampholytes (pI 4–8) in the first dimension. The proteins were visualized by silver staining, as described under "Experimental Procedures." *B*, detection of biotinylated surface proteins from the A549 cell line. Intact A549 lung adenocarcinoma cells were subjected to surface biotinylation, as described under "Experimental Procedures." Solubilized proteins from the biotinylated cells were resolved by two-dimensional PAGE using carrier ampholytes (pI 4–8) in the first dimension, then transferred to PVDF membranes. The biotinylated proteins were visualized by hybridization with streptavidin-HRP complex. *Arrows* point to biotinylated proteins that were identified by mass spectrometry. Interestingly, the biotinylated proteins (*B*) are not visualized in the silver stain image of the same lysate (*A*).

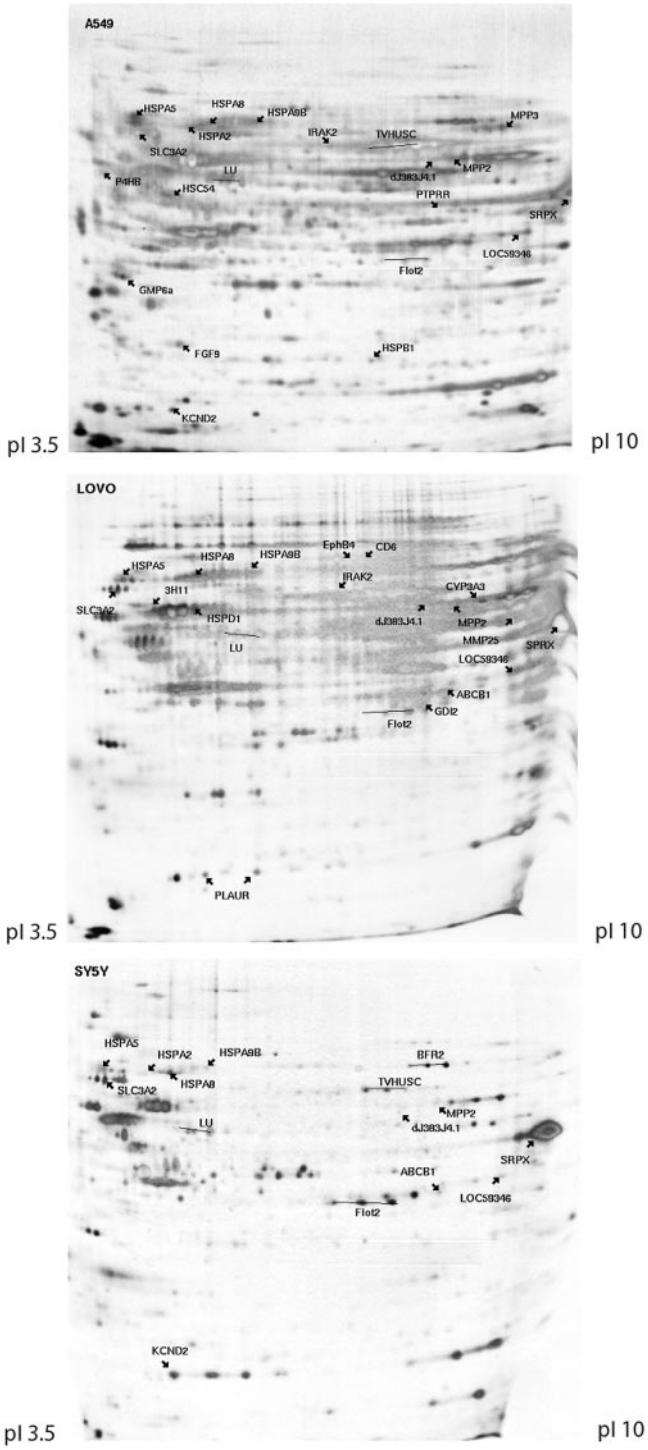

FIG. 2. **Visualization of purified biotinylated surface proteins isolated from the A549, LoVo, and SH-SY5Y cell lines.** Surface proteins of the A549, LoVo, and SH-SY5Y cell lines were biotinylated and purified as described under "Experimental Procedures." Following solubilization, the proteins were resolved by two-dimensional PAGE using IPG in the first dimension then visualized by mass spectrometry-compatible silver staining, as described under "Experimental Procedures." *Arrows* point to biotinylated proteins that were identified by mass spectrometry.

Identification of Biotinylated Surface Membrane Proteins—We sought to identify the biotinylated proteins we considered to be abundant, based on their intensity in images of two-dimensional blots that were hybridized with streptavidin/horseradish peroxidase. These proteins also occurred as relatively abundant proteins in two-dimensional silver stain patterns of proteins eluted from monomeric avidin columns. Preparative quantities of biotinylated surface membrane proteins from $\sim 3 \times 10^8$ cells were prepared for each of the cell lines analyzed. Following solubilization, the biotinylated proteins were captured and purified on

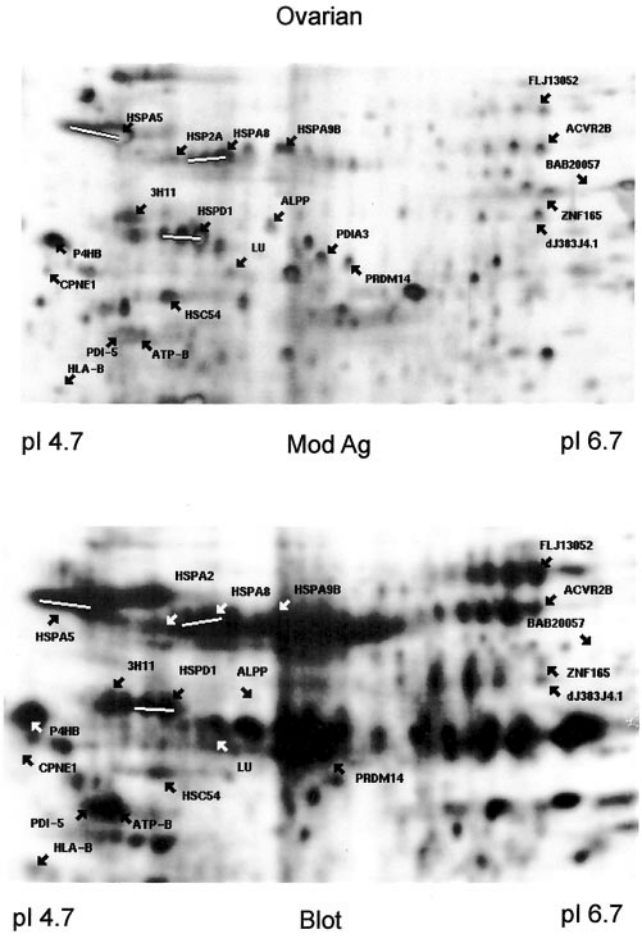

FIG. 3. **Similarity of ovarian biotinylation patterns as visualized by hybridization and silver-stained images of the same monomeric avidin column eluate.** Surface proteins of ovarian cells were biotinylated and purified as described under "Experimental Procedures." Following solubilization, the proteins were resolved by two-dimensional PAGE using carrier ampholytes (pI 4–8) in the first dimension then visualized either by mass spectrometry-compatible silver staining or hybridization with streptavidin-HRP complex, as described under "Experimental Procedures." *Arrows* point to biotinylated proteins that were identified by mass spectrometry. Interestingly, the patterns visualized by silver stain and hybridization appear to be virtually identical.

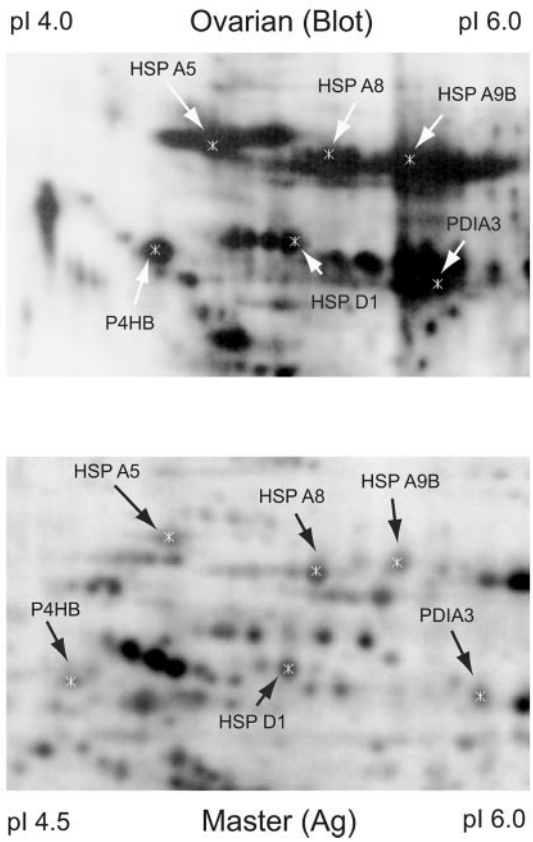

FIG. 4. **Comparison of the ovarian biotinylation patterns to a lung adenocarcinoma master image.** Surface proteins of ovarian cells were biotinylated and purified as described under "Experimental Procedures." Following solubilization, the proteins were resolved by two-dimensional PAGE using carrier ampholytes (pI 4–8) in the first dimension then visualized either by hybridization with streptavidin-HRP complex, as described under "Experimental Procedures." The image was compared with a silver stained image of a lung adenocarcinoma, which serves as a data base master image. *Arrows* point to biotinylated proteins that were found to be in common between the two images and that have been identified by mass spectrometry.

monomeric avidin columns. The eluted proteins were resolved by two-dimensional PAGE, after which the gels were stained with a mass spectrometry-compatible silver stain. One unstained gel from each preparation of silver-stained gels for protein identification was transferred to a PVDF membrane and hybridized with streptavidin/horseradish peroxidase complex to visualize the proteins that were biotinylated. In comparison to databases of proteins previously identified in our laboratory, we noted that some of the visualized biotinylated proteins actually represent proteins that were previously identified in whole cell lysates. However, considerable enrichment was evident in the biotinylated surface protein patterns (Fig. 4).

We next determined whether patterns obtained from the various cell populations (*e.g.* A549 lung adenocarcinoma, papillary ovarian carcinoma, SH-SY5Y neuroblastoma, LoVo colon carcinoma, and acute lymphoblastic leukemia-B cell type) were sufficiently distinctive to identify surface proteins with restricted expression patterns. Although it was apparent that some proteins were expressed in all cell types, some of the visualized biotinylated proteins were detected in some cell types but not others (Table I). To verify that proteins considered to be similar in different cell types based on pattern matching were indeed similar, we excised spots that were evident in two or more cell types from gels prepared from each of the cell types. These proteins were subjected individually to mass spectrometric identification, which confirmed that they had the same identity (Table I). For example, the sushi-repeat-containing protein was identified in the A549 lung adenocarcinoma cell line (Fig. 2), and its presence was confirmed in both the LoVo colon carcinoma and SH-SY5Y neuroblastoma cell lines, as predicted based on spot matching. The sushi-repeats are characteristic motifs in members of the selectin family of cell membrane proteins with functional roles in cell adhesion (15, 16). Additionally, we identified the Lutheran blood group glycoprotein in both the A549 and in the SH-SY5Y cell lines (Fig. 2). The Lutheran glycoprotein, a membrane protein belonging to the immunoglobulin superfamily, has been shown to act as a specific receptor for laminin in cell-matrix interaction (17). Furthermore, the 4F2 heavy chain antigen (also known as CD98) was identified in SH-SY5Y, A549, LoVo, and the Sup-B15 leukemia cell lines. *In vitro* binding studies have shown that the 4F2 heavy chain (CD98) interacts specifically with integrin β1A (18), and it has been implicated in cell activation and proliferation in various cancer cells, including those of colon, breast, and lung (19). Moreover, there is a recent report demonstrating that a specific antibody targeting the 4F2 heavy chain antigen inhibited the growth of tumor cells that expressed the antigen (20).

TABLE I

Identified surface proteins from the various cancer cell types

Proteins identified by cell surface biotinylation were subjected to mass spectrometry. They are indicated (MS) according to the cell type(s) that they were identified in. *Presence* or *Absence* indicates whether the protein spot was identified in other cell types by gel matching. The presence of a putative signal peptide is indicated.

	Gene symbol	Gene index number	SY5Y	A549	LoVo	Ovarian	ALL-B cell	Signal peptide
Sushi-repeat-containing protein	SRPX	gi:2498958	MS	MS	MS	Absent	Absent	Yes
Similar to PDZ-LIM Protein Mystique	LOC59346	gi:21361888	Present	MS	Present			
MAGUK P55 subfamily member 3	MPP3	gi:2497512	Absent	MS	Present			No
Lutheran blood group glycoprotein	LU	gi:1708887	MS	MS	Present	Present	Absent	No
Protein tyrosine phosphatase receptor type R	PTPRR	gi:2078323	Absent	MS		Absent	Absent	No
Voltage-sensitive potassium channel	KCND2	gi:9789986	MS	MS	Absent		Absent	Yes
4F2 antigen Heavy chain	SLC3A2	gi:177207	Present	MS	Present	Absent	Present	No
Protein-tyrosine kinase src, neuronal	TVHUSC	gi:625219	MS	MS				No
Multidrug resistance protein (P-glycoprotein)	ABCB1	gi:4505769	MS	Absent	MS	Absent	Absent	No
Rab GDP dissociation inhibitor Beta	GDI2	gi:6958323	Absent	Absent	MS			No
Cytochrome P450 nifedipine	CYP3A3	gi:510086	Absent	Absent	MS			No
MAGUK p55 subfamily member 2	MPP2	gi:2497511	Present	Present	MS	Absent	Absent	No
Interleukin-1 receptor associated kinase-2	IRAK2	gi:12230224	Absent	Present	MS		Absent	No
CD6	CD6	gi:13637684	Absent	Absent	MS			No
Ephrin type-B receptor 4	EphB4	gi:13279062	Absent	Absent	MS			No
Colon tumor antigen 3H11		gi:12711598	Absent	Absent	MS	Present	Present	No
60-kDa heat-shock protein	HSPD1	gi:129379	Present	Present	MS	MS	Present	No
u-plasminogen activator receptor form 2	PLAUR	gi:4335704		Absent	MS		Absent	Yes
Membrane type matrix metalloproteinase 6	MMP25	gi:12585274			MS			Yes
78-kDa glucose-regulated protein, BiP	HSPA5	gi:14916999	Present	Present	Present	MS	Present	No
Heat-shock 70-kDa protein 2	HSPA2	gi:1708307	Present	Present		MS	Present	No
GRP75	HSPA9B	gi:21264428	Present	Present	Present	MS	MS	No
Kelch-like protein X	dJ383J4.1	gi:12314036	Present	Present	Present	MS	Present	No
Protein disulfide isomerase	P4HB	gi:339647		Present		MS	Present	Yes
Alkaline phosphatase, placental	ALPP	gi:130738		Absent		MS	Present	Yes
Heat-shock cognate protein, 54 kDa	HSC54	gi:11526572		Present		MS	Present	No
Cation-dependent mannose-6-P receptor	M6PR	gi:106962				MS		Yes
Membrane glycoprotein M6-a	GPM6a	gi:2506889		Present		MS	Present	No
Membrane progesterone receptor component 1	PGRMC1	gi:5729875				MS	Present	No
Heat-shock 27-kDa protein 1	HSPB1	gi:4504517		Present		MS		No
Heat-shock cognate 71 kDa protein	HS7C	gi:5729877				MS		No
Activin receptor type IIB precursor	ACVR2B	gi:20532386	Absent	Absent	Absent	Present	MS	Yes
Fibroblast growth factor receptor 2	BFR2	gi:399110	MS	Absent	Absent		Absent	Yes
Flotilin 2	FLOT2	gi:13277550	MS	Present	Present	Absent	Absent	No

We have also identified biotinylated surface membrane proteins that were considered to be abundant but whose corresponding spots were found in only one or two of the cell types examined. Among these were a protein tyrosine phosphatase receptor type R, found in a spot that occurred only in A549 cells (21), and the urokinase plasminogen activator receptor 2 (CD87), found in a spot that occurred only in LoVo cells. It has been suggested that CD87, a glycosylphosphatidylinositol-anchored membrane receptor, is involved in both plasminogen activation and cellular adhesion (22). Additionally, a spot, found in both ovarian tumor cells and in Sup-B15 acute lymphocytic leukemia-B cells but not in the other cells examined, was identified as the activin receptor type IIB. Activin is well known for its inhibitory regulation of cell proliferation, enhancement of apoptosis, and suppression of cancer formation and progression. Aberration in the activin signaling pathway has been considered a possible cause of malignant transformation in various tissues, including breast cancer, prostate cancer, and leukemia (23).

Importantly, we also found that the spot identified as corresponding to Ephrin type B receptor 4, a member of a large family of receptor tyrosine kinases, was detectable in the LoVo cell line, but not in other cell lines examined. The Ephrin type B receptor 4 and its ligand Ephrin B2 appear to provide critical guidance cues at points of cell-cell contact in cardiovascular development. In adult settings of neo-angiogenesis, such as in tumors, it appears that the endothelium of a subset of new vessels strongly expresses the Ephrin B2 ligand (24), whereas the tumor cells express both the ligand and the receptor (25, 26). Interestingly, it has been demonstrated that the Ephrin type B receptor 4 is up-regulated in colon tumors when compared with normal colon tissue from the same patient (26). As such, the Ephrin type B receptor 4 may provide angiogenic signaling for the recruitment of new blood vessels to support tumor growth. To confirm the restricted expression of the Ephrin receptor, we examined whether we could localize this protein in the plasma membrane of both the LoVo and A549 cell lines by indirect immunofluorescence microscopy. We found that the Ephrin receptor was present on the surface of LoVo but not on the surface of A549 (Fig. 5), in confirmation of the results that we obtained by analysis of biotinylated proteins.

Identification of Chaperone Proteins at the Cell Surface—Remarkably, we also found that a relatively large set of proteins with chaperone function, including heat-shock proteins, was highly abundant on the cell surface (Fig. 6). These proteins include GRP78, GRP75, HSP70, HSP71, HSP60, HSP54, HSP27, and protein disulfide isomerase. Protein disulfide isomerase is an endoplasmic reticulum protein that catalyzes protein-folding reactions and increases the rate at which proteins attain their final folded conformation. GRP78 (BiP) is an endoplasmic reticulum (ER) chaperone whose function is generally thought to be limited to the structural maturation of nascent polypeptides and helps to prevent protein-folding intermediates from aggregating and stabilize energetically unfavorable conformations of polypeptides to minimize irreversible protein misfolding (27). We found, by analysis of biotinylation patterns, that GRP78 was expressed on the surface of all cell types analyzed in this study. To confirm the ubiquitous expression of GRP78, we examined whether we could localize this protein to the plasma membrane of the SH-SY5Y, LoVo, and A549 cell lines and the ovarian tumor cells by indirect immunofluorescence microscopy. We found that GRP78 was present

on the surface of all four cell lines examined (Fig. 7), in confirmation of the results that we obtained by analysis of biotinylated proteins.

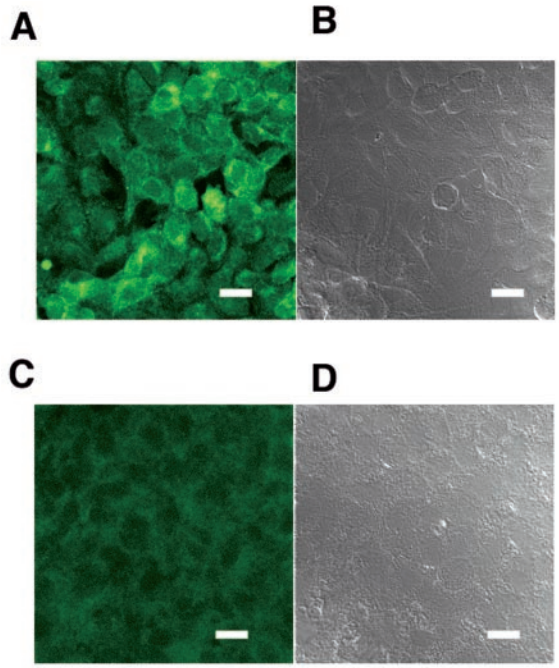

FIG. 5. **The Ephrin B4 receptor is expressed in a restricted pattern between the LoVo and A549 cell lines.** LoVo colon carcinoma and A549 lung adenocarcinoma cells were plated and grown for 48 h then fixed and stained for immunofluorescence with Rabbit anti-EphB4 antibodies, as described under "Experimental Procedures." Identical fields are shown for both the immunofluorescence and the transmitted light image (differential interference contrast). The LoVo cell line is shown in *A* and *B*. The A549 cell line is shown in *C* and *D*. *Bar*, 20 μm.

HSP70 was originally thought to be ubiquitously expressed as a cytoplasmic protein whose function was to capture folding intermediates to prevent protein misfolding and aggregation and to facilitate proper refolding (28–30). We found by analysis of biotinylation patterns that HSP70 was expressed on the surface of all cell types analyzed, which we confirmed by indirect immunofluorescence microscopy with LoVo cells (Fig. 8).

Correlations with RNA Levels—Comparison of the biotinylation patterns between the various cell types analyzed revealed that some biotinylated protein spots exhibited a lineage-restricted expression. Importantly, however, migration of biotinylated protein may vary (both by pI and molecular weight) between cell lines, dependent upon various post-translational modifications (*e.g.* glycosylation, phosphorylation, and sulfation). To determine the cell line expression of genes corresponding to the biotinylated proteins that were identified by mass spectrometry, high density oligonucleotide microarrays were utilized to generate gene expression profiles for all cell types examined. Although not all identified proteins were represented on our microarrays, in some cases the mRNA expression data was concordant with the restricted expression of the corresponding protein (Fig. 9). For example, the sushi-repeat-containing protein was found to be present in the A549, LoVo, and SH-SY5Y cell populations, but not in the ovarian or the ALL-B cells, by both mRNA expression and surface membrane profiling. Moreover, the multidrug resistance protein (P-glycoprotein) was found to be present in the SH-SY5Y and LoVo cell lines, but absent in the A549 cell line, the ovarian tumor cells, and the ALL-B leukemia cells by both mRNA and surface membrane profiling. In contrast, however, the mRNA encoding flotilin 2 was expressed in all cell types examined, but the protein spot of interest was not visualized in the ovarian and the ALL-B cells (Fig. 9). This suggests that the identified isoform of flotilin 2 was expressed on the cell surface in a lineage-restricted fashion.

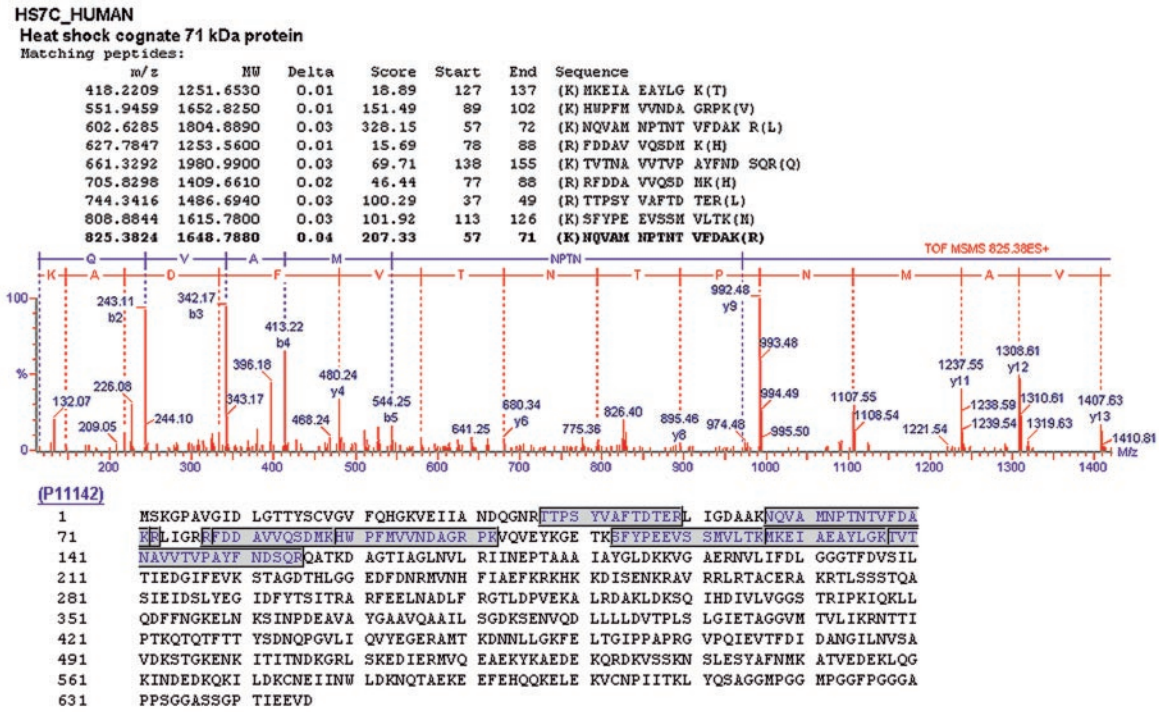

FIG. 6. **Identification of HSP71 by Q-TOF mass spectrometry.** The MS/MS spectrum of HSP71 obtained after trypsin digestion is shown by analysis with ESI-Q-TOF, coupled with nanoflow capillary high-performance liquid chromatography. The precursor ion shown in the figure is *m/z* 825.3824, and resultant peaks were searched against the non-redundant SwissProt protein sequence data base using the ProteinLynx global server. A total of nine tryptic peptides, as shown, matched the heat-shock cognate 71-kDa protein.

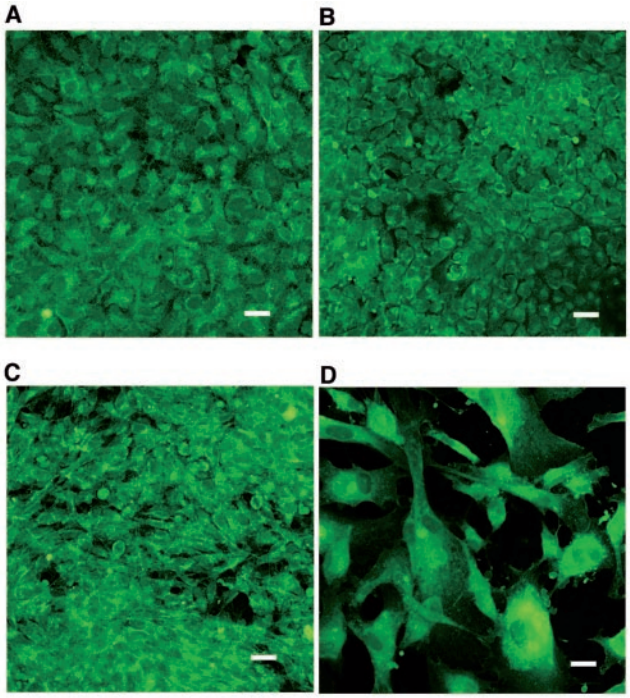

FIG. 7. **GRP78 is expressed on the cell surface in a ubiquitous pattern.** Ovarian, SH-SY5Y, LoVo, and A549 cells were plated and grown for 48 h then fixed and stained for immunofluorescence with goat anti-GRP78 antibodies, as described under "Experimental Procedures." The A549 cell line is shown in *A*, the LoVo cell line is shown in *B*, the SH-SY5Y cell line is shown in *C*, and the ovarian cells are shown in *D*. *Bar*, 20 μm.

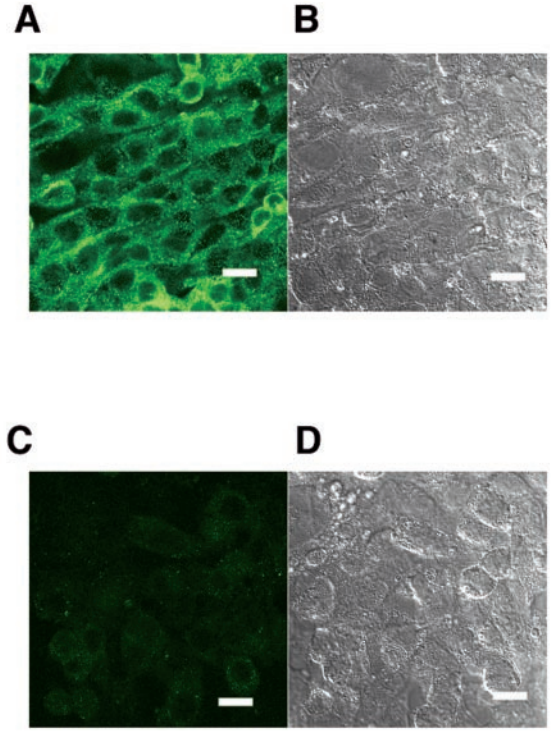

FIG. 8. **HSP70 is expressed on the cell surface of LoVo cells.** LoVo colon carcinoma cells were plated and grown for 48 h then fixed and stained for immunofluorescence with mouse anti-HSP70 antibodies, as described under "Experimental Procedures." Identical fields are shown for both the immunofluorescence and the transmitted light image (differential interference contrast). Anti-HSP70 staining is shown in *A* and *B*. The staining obtained with normal mouse IgG is shown in *C* and *D*. *Bar*, 20 μm.

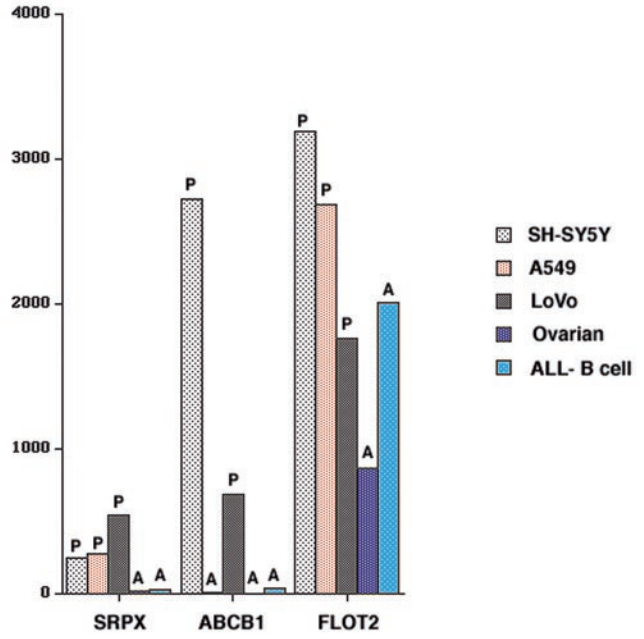

FIG. 9. **Concordance of genomic and proteomic data for SRPX, ABCB1, and FLOT2 in the various cell types examined.** mRNA expression levels were determined for SRPX, ABCB1, and FLOT2 by analysis of high density oligonucleotide microarray data, as described under "Experimental Procedures," and are shown in the graph. The presence of protein expression (*P*) or absence of protein expression (*A*) in the identified protein spot is as indicated. Interestingly, for SRPX and ABCB1 there was strict concordance between genomic and proteomic data for all cell types. For FLOT2, however, it appears by genomic data that FLOT2 is expressed ubiquitously in all of the cell types, although the protein spot of interest was absent in the ovarian and ALL-B cells.

DISCUSSION

The approach that we have developed consists of the biotinylation of plasma membrane proteins of freshly isolated cells, primary cultures or cell lines, followed by their comprehensive profiling and identification. Previously, most protein-related biotinylation applications have dealt with the isolation/enrichment or assay of individual proteins or a small number of related proteins. A basic component in a biotin-avidin-based application is the moiety to be targeted. In the case of proteins, biotinylation is done usually via the ϵ-amino group of lysine by using an *N*-hydroxysuccinimide (NHS) ester of a biotin analog. To selectively label only those lysine residues that are extracellular in orientation, non-membrane permeable biotin reagents need to be utilized to prevent the entry of biotin into the cell. Sulfo-NHS-LC biotin is water-soluble; thus it is not permeable across hydrophobic lipid bilayers and can be utilized for the selective labeling of surface membrane proteins. Other groups that have utilized sulfo-NHS-biotin for isolation of individual cell surface proteins in intact cells have found selective biotinylation of plasma membrane proteins (31, 32). Moreover, this reagent has been used to selectively label surface membrane proteins on either the apical or basolateral surface of intact, viable epithelial monolayers, because the sulfo-NHS-biotin reagent is impermeable to tight junctions (33, 34). Importantly, calnexin, a molecular chaperone in the endoplasmic reticulum has been localized on the cell surface by use of this biotinylation reagent (35).

Several findings have emerged from our studies. We have identified both glucose-regulated proteins (GRPs) and heat-shock proteins as relatively highly abundant proteins on the cell surface of a wide variety of cell types. These proteins were originally identified as being either cytoplasmic (28, 30) or endoplasmic reticulum (36) proteins. However, it has recently

been demonstrated that HSP70 does occur on the surface of a number of cell types (37–48). However, our ability to comprehensively profile cell surface proteins has uncovered the high abundance of heat-shock proteins compared with other cell surface proteins. It is interesting to note that recent data have implicated the endoplasmic reticulum (ER) as a membrane source. Fusion of the ER with the macrophage plasmalemma, underneath phagocytic (49) cups, has been found to be a source of membrane for phagosome formation in macrophages (50).

The functionality of heat-shock proteins at the cell surface has begun to be elucidated, with recent work focusing on heat-shock protein-receptor interactions. Heat-shock proteins derived from tumor cells are recognized by T cells with either T cell receptor, α,β or γ,δ (49). It has been shown that heat-shock proteins are linked to TLRs (toll-like receptors) as, unlike in wild type macrophages, HSP60 failed to activate TLR4-defective macrophages (51). Moreover, it has been demonstrated that the heat-shock protein Gp96-TLR2/4 interaction results in activation of NFκB-driven reporter genes and mitogen- and stress-activated protein kinases (52). Additionally, HSP70 also activates the IL-1 receptor signaling pathway (53). Furthermore, it has been shown that a signaling complex of receptors, comprising heat-shock proteins 70 and 90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5, is formed during immune system recognition of bacterial lipopolysaccharide (54). Thus, it has been demonstrated that heat-shock proteins and some receptors interact at the cell surface to stimulate receptor-mediated functions.

It is likely that proteins that are routinely found in the endoplasmic reticulum lumen follow the classic secretory pathway out of the cell. However, a number of the proteins that we have identified on the cell surface, including the majority of heat-shock proteins, do not encode transmembrane domains or, for that matter, signal sequences (which target the nascent polypeptide into the secretory pathway) within their genomic structure. Although it is unclear as to how these proteins were targeted to the cell surface, it is unlikely that they utilized the classic secretory pathway (ER to Golgi to plasma membrane) for their targeting. It has been demonstrated that several proteins, including basic fibroblast growth factor, interleukin-1β (IL-1β), galectin-3, thioredoxin, and HIV-Tat (55–59), are secreted in a non-classic manner, independent of the ER-Golgi pathway. Furthermore, it has been recently shown that, although exogenously expressed, properly folded green fluorescent protein is retained in the cytosol, and improperly folded green fluorescent protein is secreted from the cell cytosol through a non-classic secretory pathway (60). Although an ATP-binding cassette (ABC) transporter appears to be involved in the secretion of IL-1β (61) and a Na^+/K^+ ion channel has been implicated in the secretion of basic fibroblast growth factor (62), apparently galectin-3 is secreted from the cytosol by membrane blebbing. This last pathway appears capable of post-translational export of fully folded proteins (63). One possible explanation as to how the heat-shock proteins reached the cell surface is that they accompanied misfolded proteins, or peptide fragments out of the cytosol via a non-classic pathway. Alternatively, they may be actively transported from their site of synthesis in the cytosol to help maintain structural integrity among the individual components of various receptor complexes.

Biotinylation provides an effective tool for the detection and purification of proteins. However, to retain both biological activity and ligand-binding properties and to facilitate the identification of the labeled proteins by mass spectrometry, it is necessary to perform biotinylation reactions that do not extensively biotinylate proteins. Furthermore, although complete solubilization of the labeled cells is desirable, the best reagents for solubilization may interfere with the ability to capture and purify the biotinylated proteins on a monomeric avidin column. We have optimized the biotinylation approach for the analysis of surface membranes by comparing patterns obtained with different protocols for the solubilization of proteins and for the capture and purification of the biotinylated proteins. Our methodology for the identification of surface membrane proteins is based upon the successful biotinylation and subsequent purification of the biotinylated proteins on monomeric avidin columns for enrichment purposes. Knowing that the intact protein was biotinylated allows us to ascertain that the identified protein was present in the membrane compartment. Such information, which would be lacking if we subjected the complex mixture of proteins to proteolytic digestion (as is the case in a MudPIT analysis (64)) provides evidence for us too properly identify the "tagged" protein. However, it is likely that additional biotinylated proteins have failed to be adequately solubilized for their effective capture and identification. Thus, further refinements of our present strategy, such as by partial digestion of biotinylated proteins that fail to be solubilized with our present mixture, may further increase the repertoire of biotinylated proteins detected on the cell surface.

The approach we have implemented has wide applicability to many different types of cells, bacteria, or subcellular fractions. A recent study has been reported using a similar biotinylation approach for the identification of surface membrane proteins isolated from *Helicobacter pylori* (10). Some 82 biotinylated proteins were resolved by two-dimensional PAGE, of which 18 proteins were identified by comparison to proteome data and by peptide mass fingerprinting. The comprehensive profiling of cell surface proteins provides an effective approach for the identification of novel targets for diagnostics and therapeutics for a wide range of diseases.

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