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Profiling of the cell surface proteome

The in depth-mining of the proteome necessitates the comprehensive analysis of proteins in individual subcellular compartments to uncover interesting patterns of protein expression that include assessment of protein location, trafficking and of post-translational modifications that are location specific. One of the compartments of substantial interest from a diagnostic and therapeutic point of view is the plasma membrane which contains intrinsic membrane proteins and other proteins expressed on the cell surface. Technologies are currently available for the comprehensive profiling of the cell surface proteome that rely on protein tagging of intact cells. Studies are emerging that point to unexpected patterns of expression of specific proteins on the cell surface, with a common occurrence of proteins previously considered to occur predominantly in other compartments, notably the endoplasmic reticulum. The profiling of the cell surface and plasma membrane proteomes will likely provide novel insights and uncover disease related alterations.

Keywords: Biotinylation / Chaperon proteins / Leukemia / Mass spectrometry / Plasma membrane proteins
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1 Introduction

The plasma membrane is a sub-cellular compartment of substantial interest in regard to various aspects of disease, from molecular diagnosis to therapeutics. Numerous cell surface proteins represent therapeutic targets. For example, CD40, a cell surface molecule, is expressed in B-cell malignancies and in many different solid tumors. It is capable of mediating diverse biological phenomena such as the induction of apoptosis in tumors and stimulation of the immune response. It has thus been investigated as a possible target for anti-tumor therapy utilizing an agonistic anti-CD40 monoclonal antibody(mAb) or the soluble form of CD40L [1]. Also, the human epidermal growth factor receptor 2(HER2) promotes cell growth and tumor development. HER2 protein overexpression is observed in 25% to 30% of primary breast cancers and is associated with shorter survival [2]. Trastuzumab is a humanized anti-HER2 mAb that has been shown to produce response rates of 15 to 40% when used as a single agent in patients with metastatic HER2-overexpressing breast cancer [3–4] and to prolong survival when added to chemotherapy in the first-line therapy of such patients [5]. Cell surface proteins that are restricted in their ex-

pression to specific cancer(s) or that undergo restricted tumor-specific modifications could be utilized for antibody-based therapy, vaccine development or other forms of immunotherapy. Signaling pathways regulated by surface membrane proteins or receptors could also be targeted for drug-based therapy. Thus, profiling of the cell surface proteome in diseases such as cancer can provide an effective approach for the identification of novel targets for diagnostics and therapeutics.

As there are few effective strategies for profiling surface membrane proteomes, there is little data pertaining to the comprehensive analysis of surface membrane proteins. One of the current strategies is purification of plasma membranes from crude mixtures using aqueous two-phase partition [6]. However, the limitation of this approach is cross contamination by cytosolic proteins. Another strategy is identification of surface proteins using colloidal silica particles and polymers [7]. This technique is useful for vascular targeting in cancer therapeutics because the endothelium plays a primary or secondary role in many cancers. A study of the plasma membrane proteomes of fibroblasts and mammary carcinoma cells that relied on colloidal silica to isolate the membranes revealed three groups of proteins [8]. The first group comprised between 9 and 23% of all proteins at the plasma membrane and was common to both fibroblasts and mammary carcinoma. The second group, comprising approximately 40% of plasma membrane proteins was linked to cell lineage. The third group of proteins was expressed in a cell line restricted manner, independent of

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Abbreviations: ER, endoplasmic reticulum; IL-1, interleukin-1

cell lineage. Several proteins were identified that were associated with a metastatic cell line analyzed and have functions that included cell adhesion and the control of oxidant stress.

Very recently, a global surface protein biotinylation strategy, coupled with the use of MS was applied to *Helicobacter pylori* [9] and a variety of cancer cell types, identifying in the latter case an abundance of proteins with chaperone functions [10]. 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 [11–15]. Biotinylation reagents provide the tag that transforms poorly detectable molecules into probes that 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 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.

2 Materials and methods

2.1 Cell culture

The non-adherent human Leukemia cell lines, Sup B-15 (acute lymphoblastic leukemia-B cell), KG1 (acute myelogenous leukemia), U937 (acute Monoblastic leukemia), HL-60 (acute Promyelocytic leukemia) were grown at 37°C in a 6% CO₂-humidified incubator in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen, USA).

2.2 Biotinylation of membrane proteins

Cultured cells grown in 225 cm² tissue culture flasks were harvested by centrifugation (1000 × *g*, 10 min at 4°C) and washed three times with RPMI-1640 (without added serum or protein). Cells were resuspended at a concentration of 2.5 × 10⁷ cells/mL in RPMI-1640 (without added serum or protein). EZ-Link Sulfo-NHS-LC-Biotin (0.5 mg/mL, Pierce, Rockford, IL, USA) was added and 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 (Invitrogen), pelleted by centrifuga-

tion and solubilized in PBS containing 2% NP-40 (Gallard/Schlessinger, Carle Place, NY, USA). The cells were further disrupted by brief sonication.

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 (US Biologicals, Cleveland, OH, USA) in PBS in order 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 followed by extensive washing with PBS. The disrupted cells with biotinylated membrane proteins 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% NP-40. The bound biotinylated proteins were eluted from the column with 5 mM D-biotin in PBS containing 1% NP-40. Fractions containing eluted protein were concentrated on Centricon YM-3 columns (Millipore, Bedford, MA, USA).

2.3 2-D PAGE

Biotinylated proteins were analyzed by 2-D PAGE. Briefly, proteins were solubilized with lysis buffer, containing 8 M urea, 2% pH 3.5–10 carrier ampholytes, 2% β-mercaptoethanol, 2% NP-40 and 10 mM PMSF. IEF was carried out using either pH 4–8 carrier ampholyte based tube gels for 13 200 Vh at room temperature. First-dimension gels were loaded onto a cassette containing the second-dimension gel, after equilibration in second-dimension sample buffer [125 mM Tris (pH 6.8) containing 10% glycerol, 2% SDS, 1% DTT and bromophenol blue]. Separation in the second dimension was performed by electrophoresis in 7–14% polyacrylamide gradient SDS gels. The samples were subjected to electrophoresis until the dye front reached the opposite end of the gel. The acrylamide used in the first dimension electrophoresis, urea, ammonium persulfate and piperazine diacrylamide were all purchased from Bio-Rad (Rockville Centre, NY, USA). The acrylamide used in the second dimension electrophoresis was purchased from Serva (Crescent Chemical, Hauppauge, NY, USA) and the carrier ampholytes were purchased from Gallard/Schlessinger. Some gels were silver-stained and digitized for pattern analysis. For some other gels, the resolved proteins were transferred

to Immobilon-P PVDF membrane (Millipore). Unstained membranes were prepared for hybridization by incubation with blocking buffer [consisting of TBS containing 1.8% nonfat dry milk and 0.1% Tween 20] for 2 h. They were then washed and incubated with a horseradish peroxidase conjugated biotin-streptavidin complex (Amersham, Arlington Heights, IL, USA) (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 (Amersham) and exposed to XAR-5 X-ray film (Amersham). Visualized patterns were directly compared to comparable gel silver-stain patterns.

3 Results and discussion

3.1 Biotinylation of surface membrane proteins

This approach for the comprehensive profiling and identification of surface membrane proteins involves the selective biotinylation of the surface proteins of intact cells. Following separation by 2-D PAGE and transfer to PVDF membranes, the biotinylated proteins are visualized by hybridization with a streptavidin/horseradish peroxidase complex. In the case of proteins, biotinylation is usually

performed with the ϵ -amino group of lysine 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 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.

Figure 1A displays the biotinylated proteins in whole cell lysates of U937 acute monoclastic leukemia cells following cell surface labeling. It is evident that the pattern of visualized biotinylated proteins is quite rich in separated proteins which are not visualized in silver-stained 2-D gels of the same whole cell lysates (Fig. 1B). 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). While some proteins could be matched in their location between the silver stained (Fig. 1B) and biotinylated (Fig. 1A) 2-D patterns, most biotinylated proteins had no match in the silver-stained 2-D pattern of whole cell lysates. This strongly suggests that we are able to obtain a selective and enhanced visualization of low abundance proteins through biotinylation of the surface membrane.

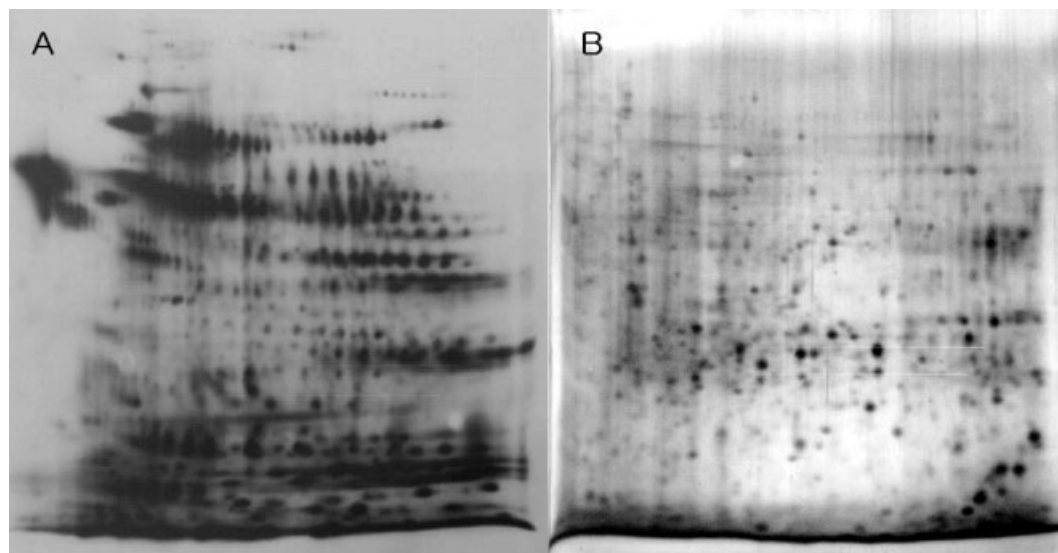


Figure 1. Visualization of biotinylated surface proteins in U937 acute monoclastic leukemia cells. A. Detection of biotinylated surface proteins from U937 cells. Surface proteins from intact U937 cells were biotinylated, solubilized, resolved by 2-D PAGE, and then transferred to PDVF membranes. They were visualized by hybridization with streptavidin-HRP complex. Interestingly, most proteins detected, were not detectable in 2-D gels of the same whole cell lysates. B. 2-D PAGE analysis of U937 cellular proteins. Proteins from U937 cells were solubilized and resolved by 2-D PAGE using IPG in the first dimension.

3.2 Purification of biotinylated surface membrane proteins

As the population of surface membrane proteins that are biotinylated represent low abundance proteins at the whole cell level, it is necessary to utilize an enrichment procedure following biotinylation to allow identification of the biotinylated proteins. To this end, a column of immobilized monomeric avidin is utilized to bind biotinylated proteins. They are subsequently eluted from the column in PBS containing 5 mM D-biotin and 1% NP-40. Column eluates from different cell populations are concentrated approximately 400-fold, and aliquots resolved by 2-D PAGE, followed by either silver staining for some aliquots or transfer to PVDF membranes for others. PVDF membranes are hybridized with streptavidin/horseradish peroxidase and visualized with ECL reagents. 2-D patterns for eluted proteins visualized by silver staining are remarkably similar to 2-D patterns of proteins transferred to PVDF membranes, then hybridized with streptavidin/horseradish peroxidase and detected with ECL reagent, indicating that most proteins captured on the column represented biotinylated proteins. Figure 2 shows that the pattern of biotinylated proteins from the U937 cell

line that were visualized by hybridization was highly similar to the pattern obtained from silver-stained gels of another aliquot of the same preparation that were bound to a monomeric avidin column. Figure 3 shows that the patterns of biotinylated proteins obtained from four different leukemia cell lines shared some common proteins, while showing other proteins that were restricted to each cell population.

While 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. The biotinylation approach for the analysis of surface membranes is optimized by comparing patterns obtained with different protocols for the solubilization of proteins and for the capture and purification of the biotinylated proteins. However, it is likely that some 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 current cocktails, may further increase the repertoire of biotinylated proteins detected on the cell surface.

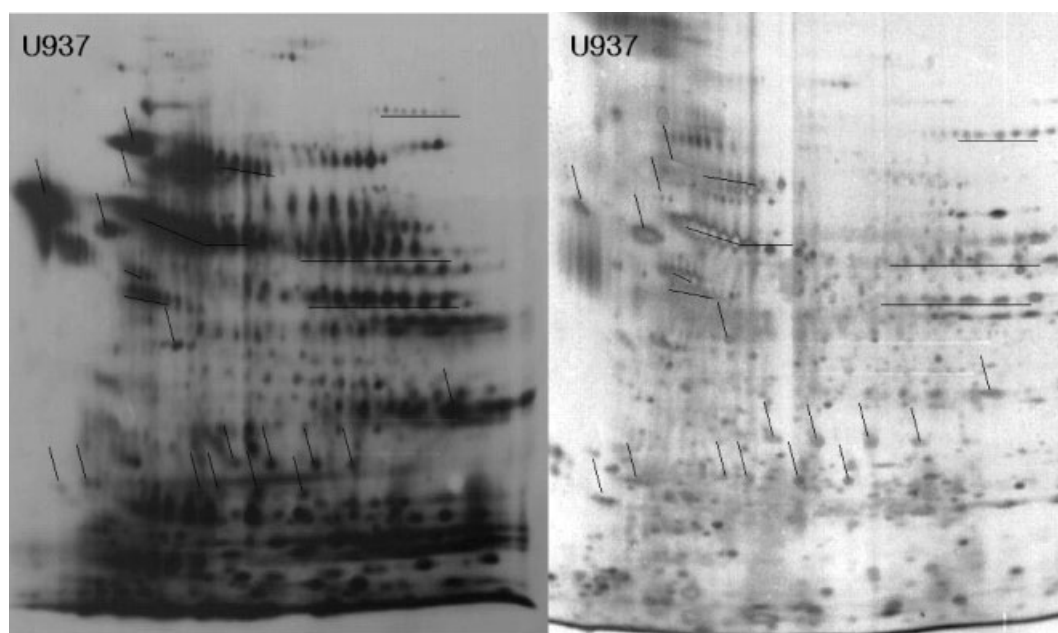


Figure 2. Similarity of the biotinylation patterns of proteins from the U937 cell line as visualized by hybridization and silver-stained images of the same monomeric avidin column eluate. Surface proteins from U937 cells were biotinylated and purified as described in Section 2.2. Following solubilization, the proteins were resolved by 2-D PAGE using carrier ampholytes (pI 4–8) in the first dimension, then visualized either by MS-compatible silver staining or hybridization with streptavidin-horseradish peroxidase complex, as described in Section 2.3. Solid lines point to biotinylated proteins that were identified by mass spectrometry. Interestingly, the patterns visualized by silver stain and hybridization appear to be virtually identical.

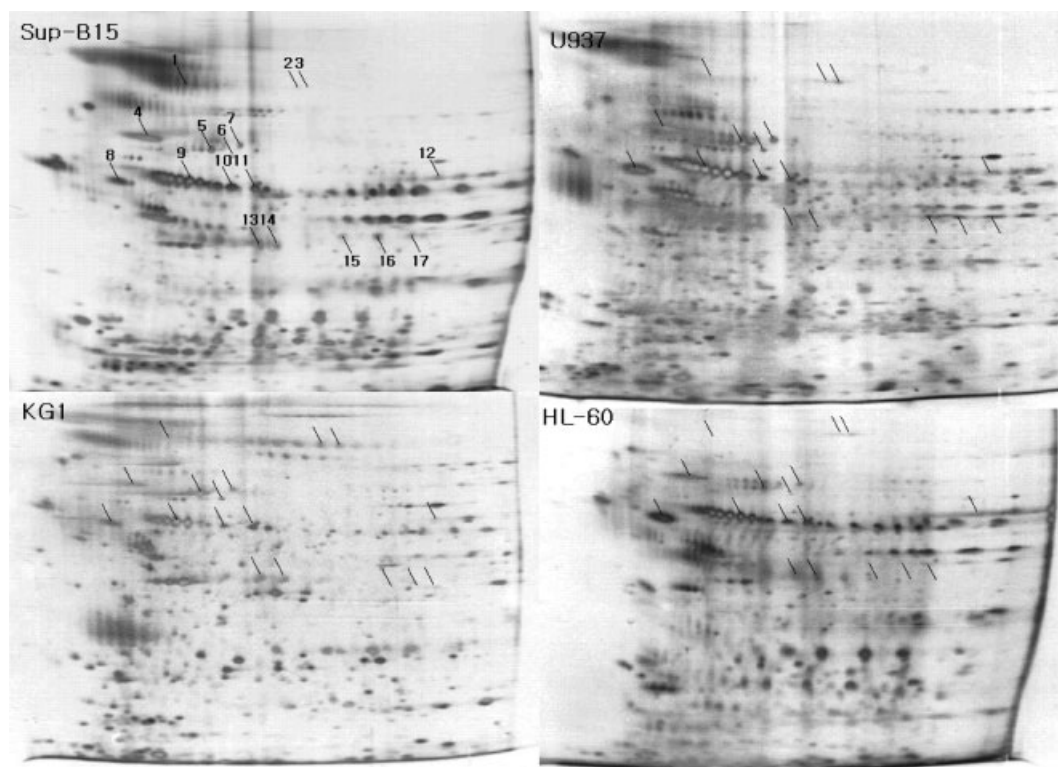


Figure 3. Visualization of purified biotinylated surface proteins isolated from four different leukemia cell lines: Sup-B15, U937, KG1, and HL60. Surface proteins from leukemic cell lines were biotinylated, solubilized, purified by avidin column, resolved by 2-D PAGE using carrier ampholytes (pI 4–8) in the first dimension, and then visualized by MS-compatible silver stain. Identified proteins are numerically labeled and indicated by lines. Solid lines point to biotinylated proteins obtained from four different leukemia cell lines which were highly similar to one another.

3.3 Identification of biotinylated surface membrane proteins

Following purification by monomeric avidin columns, the eluted proteins are resolved by 2-D PAGE, followed by staining of the gels with a MS-compatible silver-stain. One unstained gel from each preparation of silver-stained gels for protein identification is transferred to a PVDF membrane and hybridized with streptavidin/horseradish peroxidase complex in order to visualize the biotinylated proteins. Peptide mixtures are analyzed by MALDI-TOF MS or LC/MS/MS.

Figure 4 displays profiling of the surface proteome of the Sup-B15 acute B-lymphoblastic leukemia cell line. There is a relatively large set of proteins with chaperone functions, including heat shock proteins (HSPs), GRP78, GRP75, HSP70, HSP60 and PDIs (Table 1). Because these proteins were previously considered to be largely cytoplasmic [16–17] or endoplasmic reticulum (ER) proteins [18], we review our current knowledge of cell surface expression of specific chaperone proteins.

3.4 Chaperone proteins on the cell surface

PDI, previously considered to be associated with the ER, has recently been found outside the cell, either as a secreted protein or localized to the cell surface of a variety of cell types. It has been suggested that after secretion, PDI binds to the cell surface through electrostatic interactions [19]. In any case, interaction with other surface proteins is plausible, since PDI, like other members of its family, is a soluble protein, not likely to be inserted into the membrane. Some interesting roles have been attributed to cell surface PDI. In general, the protein thioredoxin is believed to be involved in the reducing activity of the cell exterior [20], so that macromolecule disulfide bridges coming into contact with or present on the cell surface can be reshuffled or reduced. Consistent with this, the cell surface thiol levels in lymphocytes and fibrosarcoma cells positively correlated with the amount of cell surface PDI [21, 22]. In contrast, an inverse correlation was found in B cell chronic lymphocytic leukemia, in which inhibition of sur-

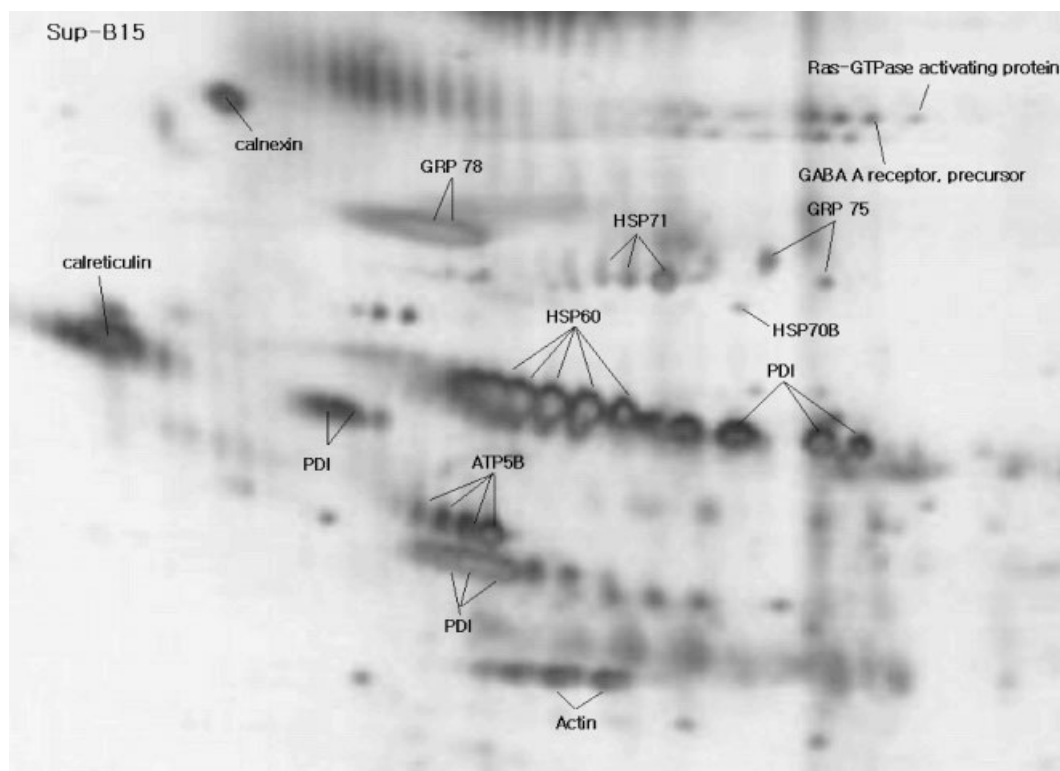


Figure 4. Identification of surface membrane proteins isolated from the Sup-B15 cell line. Purified surface membrane proteins from Sup-B15, cells resolved by 2-D PAGE and visualized by MS-compatible silver-stain, were analyzed by MALDI-TOF MS. Some of the identified proteins are marked and named with solid lines.

Table 1. Identified surface proteins from Sup-B15 acute B-lymphoblastic leukemia cell line

Number	Gene symbol	Gene index number	Protein name
1	ITGB2	14780741	Integrin beta chain, beta 2 precursor
2	TFRC	13630783	Transferrin receptor
3	TFRC	13630783	Transferrin receptor
4	HSPA5	14916999	78 kDa Glucose regulated protein, BiP
5	HSPA8	5729877	HSP 70KDa protein 8
6	HSPA2	13676857	Heat shock 70kDa protein2
7	HSPA9B	21264428	GRP75
8	PDI	2507460	Protein disulfide isomerase
9	HSPD1	129379	60 kDa heat shock protein
10	JC5704	7437388	Protein disulfide isomerase
11	JC5704	7437388	Protein disulfide isomerase
12	CAT	4557014	Catalase
13	P10319	122168	HLA-Bw58
14	P10319	122168	HLA-Bw58
15	CD38	4502665	CD38 antigen
16	CD38	4502665	CD38 antigen
17	CD38	4502665	CD38 antigen

The proteins numbered in Fig. 3. were submitted to MALDI-TOF MS analysis and identified.

face-bound PDI with bacitracin or anti-PDI antibodies resulted in an increase in thiols of the cell membrane proteins [23].

Although the function of these external thiols and their relationships with PDI remain unclear, their importance is suggested by the significant increase in surface thiols in conditions such as lymphocyte activation, leukemia, or human immunodeficiency virus (HIV)-infection [21–23].

Some interesting examples of a role for PDI in pathological events have been demonstrated. For instance, cell-surface expression of PDI has been shown to be involved in the reduction of disulfide bridges of the diphtheria toxin, enabling it to enter the cell and initiate its cytotoxic activity [20]. In addition, the infectivity of viruses, such as the Sindbis virus [24] and HIV [25], appears to depend on the reducing activity of the cell surface. In the case of HIV, a detailed study has shown the reducing agent to be PDI. Inhibitors of this enzyme such as bacitracin, an anti-PDI antibody and the membrane impermeant 5,50-dithiobis (2-nitrobenzoic acid) do not allow entry of the virus into human lymphoid cells, although CD4 receptor binding still takes place. HIV entry is dependent on the disulfide bridge reduction of the viral envelope glycoprotein gp120 [25, 26].

GRP78 (BiP) is an ER chaperone whose function was thought to be limited to the structural maturation of nascent polypeptides. It helps prevent protein-folding intermediates from aggregating and stabilizes energetically unfavorable polypeptide conformations to protect against irreversible protein misfolding [27].

Similarly, HSP70 was first thought to be ubiquitously expressed as a cytoplasmic protein whose function was to capture folding intermediates to avoid protein misfolding and aggregation and to assist in proper refolding [16, 17, 28]. The role of HSPs on the cell surface is becoming better appreciated, with recent work focusing on HSP-receptor interactions. HSPs derived from tumor cells are recognized by T cells using either T Cell Receptor α , β or γ , δ [29]. It has been shown that HSPs are linked to TLRs (toll-like receptors) since, in contrast to wild-type macrophages, HSP 60 failed to activate TLR4-defective macrophages [30]. Moreover, it has been shown that the interaction between the heat shock protein Gp96 and TLR2/4 results in activation of NF κ B-driven reporter genes and mitogen- and stress-activated protein kinases [31]. Additionally, HSP70 activates the interleukin-1 (IL-1) receptor signaling pathway [32]. Further, it has been shown that a signaling complex of receptors comprising HSPs 70 and 90, chemokine receptor 4 and growth differentiation factor 5 is formed during immune system recognition of bacterial lipopolysaccharide [33]. Thus it has been

established that HSPs and some receptors interact at the cell surface in order to stimulate receptor-mediated functions.

It is likely that proteins that are routinely found in the ER lumen follow the classical secretory pathway out of the cell. However, a number of the proteins that we have identified on the cell surface, including the majority of HSPs, do not encode transmembrane domains or, for that matter, signal sequences (which target the nascent polypeptide to the secretory pathway) within their genomic structure. While it is unclear how these proteins were targeted to the cell surface, it is unlikely that they utilized the classical secretory pathway (ER to Golgi to Plasma Membrane) for their targeting. It has been demonstrated that several proteins including basic Fibroblast Growth Factor (FGF), IL-1, galectin-3, thioredoxin and HIV-Tat [34–38] are secreted in a non-classical manner, independent of the ER-Golgi pathway. Further, it has been recently shown that while exogenously expressed, properly folded green fluorescent protein is retained in the cytosol, improperly folded green fluorescent protein is secreted from the cell cytosol through a non-classical secretory pathway [39]. An ATP-binding cassette transporter appears to be involved in the secretion of IL-1 β [40] and a Na⁺/K⁺ ion channel has been implicated in the secretion of basic FGF [41], while galectin-3 is apparently secreted from the cytosol by membrane blebbing. This last pathway appears capable of post-translational export of fully folded proteins [42]. One possible explanation as to how the HSPs reach the cell surface is that they accompany misfolded proteins or peptide fragments out of the cytosol via a non-classical 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. Finally, it is possible that the HSPs themselves may have attained a misfolded conformation in the cytosol, thus being removed from the cytosol through a non-classical pathway.

Although the functions of some chaperone proteins on surface membrane has been demonstrated, a satisfactory explanation of their function remains to be elucidated.

4 Concluding remarks

The 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. It is likely that characterization of the changes that occur at the cell surface during differentiation in response to var-

ious stimuli and during disease processes will lead to a better understanding of these processes and further expand the repertoire of diagnostic and therapeutic target.

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