ABSTRACT

Title of Dissertation: PROTEOMIC ANALYSIS OF PLASMA

MEMBRANE PROTEINS FROM DRUG

SUSCEPTIBLE AND DRUG RESISTANT BREAST

CANCER CELL LINES

Amir Mikel Rahbar, Doctor of Philosophy, 2004

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Drug discovery is an important field of research in the biotechnology and

pharmaceutical industries. Plasma membranes are rich in drug targets and other

proteins responsible for cell signaling, transport, signal transduction, and other

cellular functions. Information obtained about these proteins, and the pathways they

participate in, helps to facilitate the drug discovery process. Although these plasma

membrane proteins play important roles in cellular function, they are usually

expressed in very low abundance and are therefore hard to identify and analyze.

Comparative proteomic analysis of plasma membranes in different types of cells or

different disease states of the same cell or tissue type can help to design targeted

therapies specific to particular cell or tissue types and can be used in the identification

of biomarkers for early disease detection. In order to be able to identify proteins in the

plasma membrane it is important to start out with a plasma membrane fraction that is free of contamination from other more abundant proteins from other portions of the cell. 2D gel electrophoresis is the primary protein separation tool for use with proteomics and drug discovery, however, the inability of membrane proteins to be separated using isoelectric focusing, which is the first step in the 2D gel protocol, excludes 2D gel electrophoresis as a viable technique for the separation of membrane proteins.

This thesis develops and evaluates a method to identify proteins found in the plasma membranes of mammalian cells using a modified form of the cationic colloidal silica technique for plasma membrane isolation combined with analysis of these proteins using mass spectrometry. This method is then used in combination with metabolic stable isotope labeling to identify protein expression changes between the mitoxantrone drug susceptible and drug resistant MCF-7 breast cancer cell lines.

PROTEOMIC ANALYSIS OF PLASMA MEMBRANE PROTEINS FROM DRUG

SUSCEPTIBLE AND DRUG RESISTANT BREAST CANCER CELL LINES

by

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A. Cancer and Drug Resistance

According to the National Vital Statistics Report, in the United States, cancer has become the second leading cause of death among all ages, and was found to be the leading cause of death for those between the ages of 44 and 65.[1] As individuals increase in age, their chances of developing some form of cancer increases. Despite new therapies and lower death rates for cancer sufferers, the incidence of cancer is predicted to increase in the coming years due to the rise in average expected human lifespan and subsequent increasing aging population, and this is predicted to increase the burden of cancer on our country.[2]

Common cancer treatments are chemotherapy, radiation therapy, and surgery, and these treatments are often used in combination with one another depending upon the progression and type of cancer diagnosed.[3; 4; 5] Drugs that target DNA are among the most common type used in chemotherapy. Some of the more common drug classes are alkylating agents and antitumor antibiotics. The mechanisms these drugs use include crosslinking DNA, inducing DNA double strand breaks, intercalating into DNA, and inhibiting topoisomerases I and I. [6] The overall desired effect of these actions is to damage the DNA, interfere with the cell cycle, and ultimately induce cell death. In recent years, innovative therapies which are more specific such as agents that target DNA secondary structures, agents that target protein-DNA complexes, and

sequence specific DNA agents have been in development in order to more specifically target tumor cells.[6]

Drug resistance in cancer patients is becoming an increasingly common problem. A patient who may have responded favorably to initial treatment with a particular anti-cancer agent may have little or no response to a secondary treatment with not only the previous drug used, but also to other drugs not yet administered which can be structurally unrelated and need not have common mechanisms of action to the drug used at the onset of the illness.[7] This phenomenon is called acquired drug resistance or multi-drug resistance.[7] This multi-drug resistance is usually attributed to protein abundance changes of several classes of proteins.[7]

Figure 1 shows a representation of some of the general mechanisms of drug resistance used by the cell.[7] One way resistance is achieved is by decreasing the effective concentration of the drug in the cell. This is facilitated by decreased influx or increased efflux of chemotherapeutic agents by transporters in the plasma membrane of the cell. One method of increased efflux is to overexpress efflux transporters such as P-glycoprotein (Pgp) or Multidrug Resistance Proteins (MRP), which pump agents out of the cell.[8; 9; 10] Another method of decreasing the effective concentration in the cell is to compartmentalize the agent for later processing or removal from the cell. These mechanisms of drug resistance work well because if the drug cannot reach its target, it cannot kill the tumor cell. If the drug is
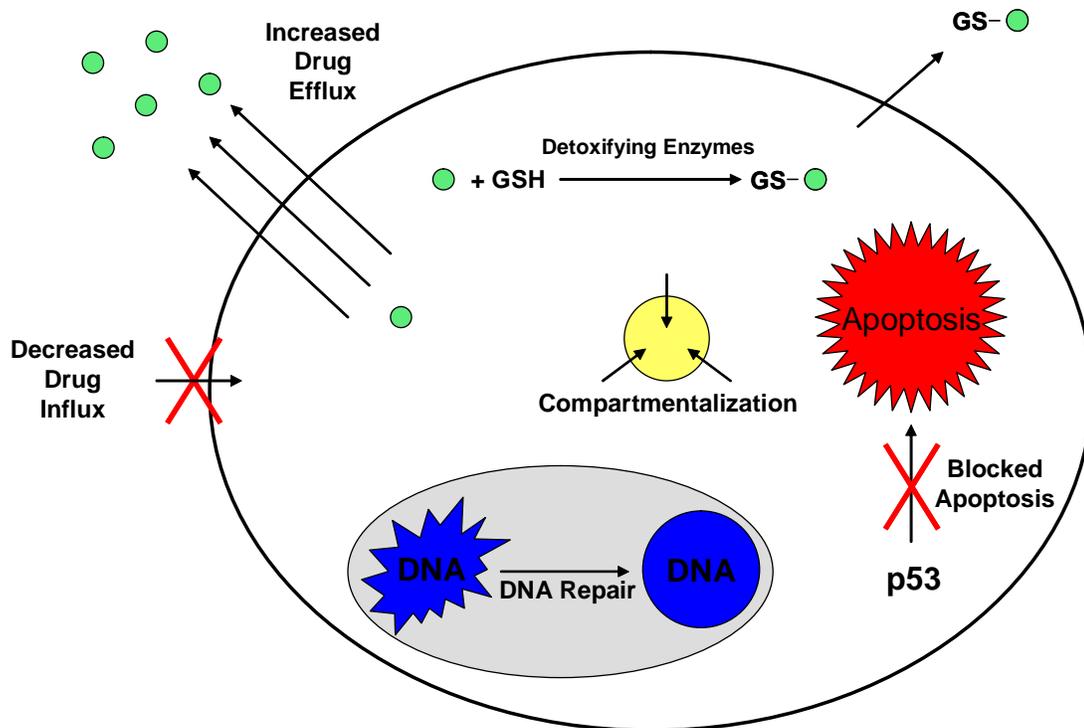
Figure 1: Mechanisms of Chemoresistance (**Gottesman et al).**[7]

allowed to accumulate in the cell, another mechanism of drug resistance is to increase the expression of phase I and phase II detoxifying enzymes such as Glutathione-S-transferase (GST) and Cytochrome P450.[11; 12; 13; 14] The cell can also increase expression of proteins which block apoptosis and allow the cell to proliferate in its mutated state. If the DNA damaging agent happens to elude all of the above mentioned mechanisms, the DNA damage done by the drug can be repaired by an increase in the expression of DNA repair proteins and the apoptotic pathway will not be activated.[7] So there are many possible cellular mechanisms working individually or in concert with one another using naturally occurring proteins and pathways which can be induced or overexpressed in order to achieve drug resistance.

B. Proteomics and Mass Spectrometry

Where a genome is the ensemble of genes possessed by an organism, a proteome can be described as the protein complement to the genome of that particular organism, or put simply, the proteome is the inventory of proteins which are being expressed by an organism under a particular set of conditions at a particular moment in time. Genomic data has a wide variety of uses and is both versatile and robust. Even before the sequencing of the human genome, genomic information could be used for identification using DNA fingerprinting. With the recent completion of the human genome sequence, we have more information available to us that can potentially help in the prevention and treatment of diseases. DNA microarrays are currently being used to study mRNA expression in different disease states, and this technique may one day be used by physicians to make quick diagnoses of genetic disorders and diseases.[15] However, post translational modifications, protein half-lives, and other protein processing events make it difficult to correlate mRNA expression with protein expression.[15; 16] For this reason determining protein expression levels directly can be a more accurate way to compare relative protein abundances.[16] Unfortunately, unlike DNA, which can be amplified by PCR, proteins cannot be amplified and detection is limited by their *in vivo* abundance, and unlike the genome, which is static, the proteome is dynamic and is dependant on various factors such as environmental stimulation and life cycle. It has been estimated that there are approximately 30,000 predicted genes in the human genome.[17] Taking into account RNA splice variants,

post translational modifications, and other post processing events, these genes are estimated to produce over 200,000 distinct proteins.[18]

The information made available by this genomic data can help in the field of proteomics. Experimental data may be compared to theoretical data calculated from an organism's genome to identify hypothetical proteins which have not been previously identified or characterized. Databases searched using bioinformatics programs often incorporate both genomic and proteomic data from previously characterized proteins.

When conducting any proteomic analysis, one is more likely to observe proteins expressed in low abundances if one minimizes the complexity of the sample. This is especially true if the target is a minor component of the sample source. For this reason, often one of the first steps in a proteomic analysis of a eukaryotic system is sub-cellular fractionation of the cells, whereby the components of the cell are separated into their individual organelles or compartments. This type of proteomic study has been called organelle-based proteomics.[18] **Table 1** showsa list of the subcellular components of an epithelial cell and what percentage each component comprises of the totalcell volume.[18] By separating organelles, chances are maximized for identification of proteins from organelles which make up a very small fraction of the cell, such as the golgi body or the plasma membrane. Without this sub-cellular fractionation, identification and analysis of proteins from whole cell lysates would only give information on the group of proteins which were the most abundant.

Organelle	% of Cell Volume
Plasma Membrane	2
Golgi	1
Endoplasmic Reticulum	15
Peroxisome	1
Lysosome	4
Mitochondria	12
Nucleus	10
Cytosol	55

Table 1: Breakdown of the subcellular components of an epithelial cell.[18]

Sometimes, as with the cytosolic sub-cellular fraction, the sample is still too complex to analyze some of the lower abundance proteins. Further separation may be required using an HPLC method such as reverse phase, size exclusion, or ion exchange chromatography. Another example of further fractionation would be to separate the soluble and insoluble fractions of a closed organelle such as the nucleus or mitochondria, which both have insoluble proteins in their membranes and soluble proteins in their aqueous interiors. Because of the limited dynamic range of proteomic techniques, sub-cellular fractionation can help to improve the number of proteins identified and characterized in samples of interest.[19; 20]

Mass spectrometry was first conceptualized in 1897 by Sir Joseph J. Thomson.[21] Since then mass spectrometry has become a versatile tool of critical importance in the field of biological research. Mass spectrometry does not actually measure the molecular mass of a molecule. It measures the mass/charge ratio of that species. In order for the molecular species to be observed it must first be ionized. Some common ionization techniques used are chemical ionization, fast atom bombardment, and electron ionization, but for the purposes of proteomics and the analysis of most biological samples, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization are the most common ionization techniques used.[22; 23; 24; 25; 26] These techniques are soft ionization techniques which allow the molecular species to become charged without much non-facilitated fragmentation of the parent species. These techniques also work well with biological samples of higher molecular masses.

MALDI ionization is a technique that uses a molecular matrix thatcan absorb energy

from a laser. A laser is directed at a cocrystallized mixture of peptides or proteins and

matrix, and the matrix absorbs the energy from the laser, causing both the matrix and

the sample to be transferred to the gas phase. The mechanism of ionization by

MALDI is not clearly understood, but the result is that the sample is ionized by H^+

transfer in the plume resulting from the absorbance of the laser energy by the

matrix.[22; 23; 27; 28] Once the sample is ionized in this manner it is moved through a

strong electric field to a mass analyzer **(Figure 2)**.[27; 28] Most of the ions created using

this technique are singly charged.

Unlike MADLI, in electrospray ionization the sample starts in the liquid phase rather

than the solid phase. The sample is usually placed in a denaturing acidic

aqueous/organic solution before ionization, typically in a ratio of 50:50:2 of methanol,

deionized water, and acetic acid respectively. The acetic acid aids in protonation of

the sample while the methanol aids in the evaporation of the liquid phase. If the

sample of interest is to be observed in its non-denatured state then an aqueous

solution such as ammonium bicarbonate can be used in place of the denaturing

methanol/water/acetic acid solution. Producing a fine spray of this sample

of highly charged droplets in the presence of a strong electric field creates ions of the

sample in the gas phase. A curtain of nitrogen gas helps to facilitate the evaporation

of the liquid and once the samples are ionized, the sample is moved into the mass
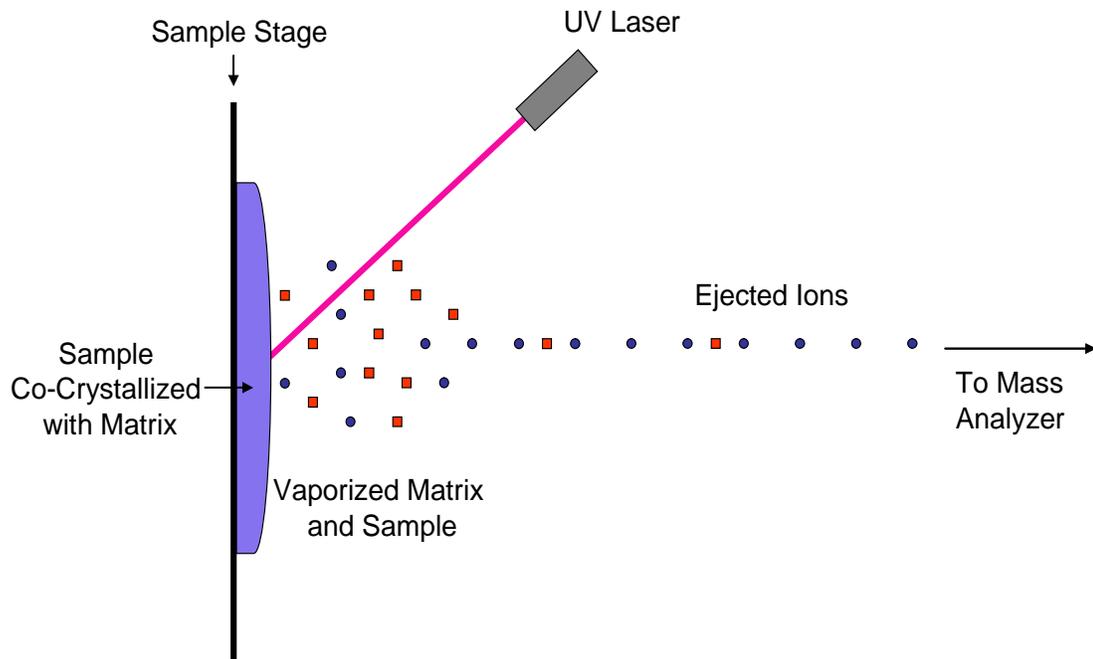
Figure 2: Schematic of the MALDI ionization method
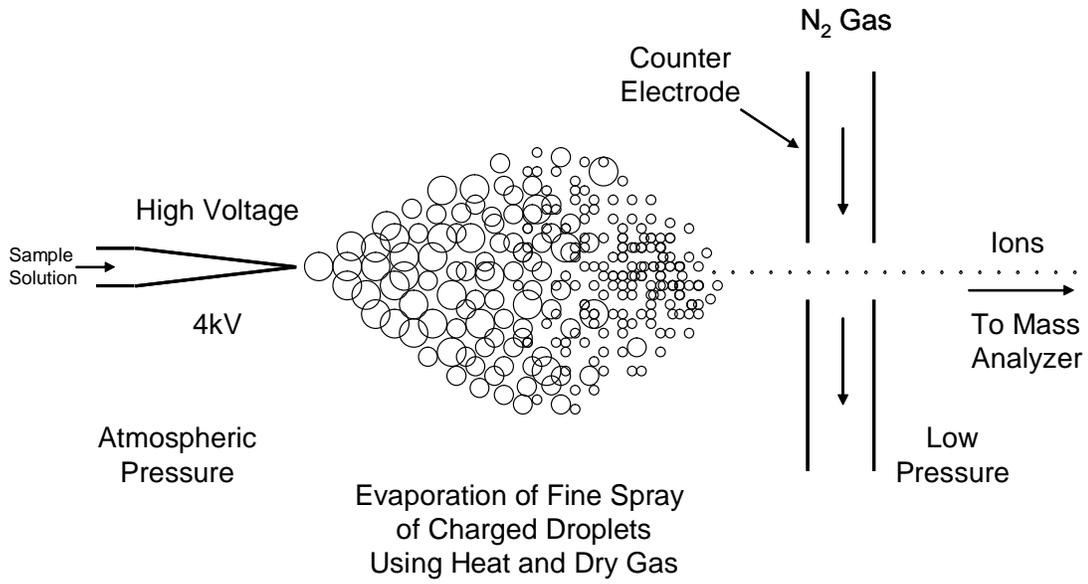
Figure 3: Schematic of the electrospray ionization method

analyzer.[25; 26; 29; 30] Electrospray ionization creates predominantly multiply charged

species, which allows the analysis of high molecular weight compounds normally

beyond the range of detection of the mass spectrometer.

There are two proteomic approaches that are used for the analysis of pr oteins using

mass spectrometry. The "top-down" approach involves the measurement of intact

protein masses using advanced mass spectrometers. The information obtained from

this type of analysis is often useful in the determination of post-translational

modifications of proteins. The intact proteins can then be fragmented and the peptides

resulting from this fragmentation can be analyzed to obtain sequence information

which can aid in the identification of the protein. This fragmentation is achieved by

collisional induced dissociation (CID), which will be discussed later.

The second proteomic approach used for the analysis of proteins is sometimes called

the "bottom-up" approach. In this method, intact proteins are subjected to enzymatic

digestion and/or chemical cleavage, and the resulting peptides are then characterized

using mass spectrometry. The information obtained from the analysis of these

peptides using mass spectrometry is then used for protein identification and analysis.

While the "bottom-up" technique is by far the more commonly used of the two

techniques, experiments have been performed using them in combination with one

another, integrating the strengths of each into the proteomic analysis.[31]

One of the more common ways to analyze proteins using the "bottom-up" method is

to digest the protein with a proteolytic enzyme and determine the molecular masses of

the resulting peptides using mass spectrometry. The set of peptide masses, along with other information such as the enzyme used and the species of origin if known, is searched against a protein database. The proteins are theoretically digested *in silico* (by the computer) and the resulting sets of molecular masses are compared to the experimental sets. From this comparison a list of possible proteins is generated with scores and probabilities to determine the confidence of the identification.[32; 33]

Proteins may undergo many post-transcript processing and posttranslational modifications, which make it difficult to predict molecular protein masses from its corresponding genomic DNA sequences. Obtaining a peptide mass fingerprint from proteins with post-translational modifications is still possible due to the fact that the intact protein is cleaved into small segments that may not contain these post translational modifications and there are usually enough unmodified peptides to obtain an identification.

A high percentage of sequence coverage is needed to obtain a reliable peptide mass fingerprint. In a situation where a positive identification cannot be obtained, microsequences from a few peptides can be provided by tandem mass spectrometry (MS/MS) for protein identification. An initial survey scan is obtained to determine the masses of the peptides introduced into the mass spectrometer and the intensities of their ions.

A peptide ion is selected from this set of peptides and allowed to pass through a mass filter and into a collision cell while all other masses are excluded. This peptide is then allowed to collide with an inert gas such as nitrogen or argon to confer vibrational excitation and induce fragmentation. This procedure is called collision induced dissociation or CID. Typically, ions are moving with low kinetic energy and the low energy collisions produce limited fragmentation along the polyamide backbone. Most of the fragmentation occurs at the amide bonds, with the predominant species from this fragmentation being b ions, where the charge is retained on the N-terminal portion of the peptide after fragmentation, and y ions, where the charge is retained on the C-terminal portion of the peptide after fragmentation (**Figure 4**).

The masses of fragment ions obtained by tandem mass spectrometry provides information about the amino acid sequence of the peptide in question. By determining the mass difference between adjacent y or b ions, the specific amino acids which make up the peptide can be determined (**Figure 5**).
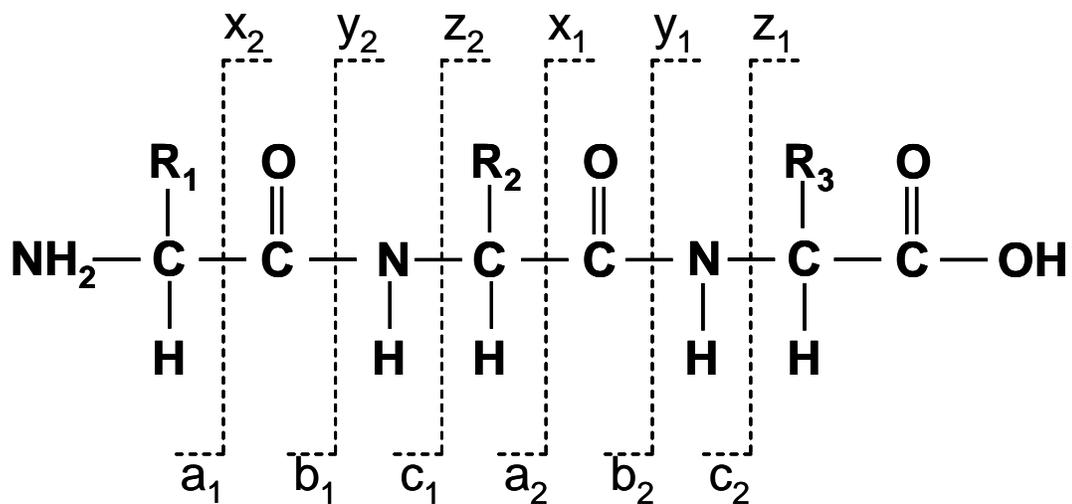
Figure 4: Fragmentation pattern of a theoretical tri-peptide.
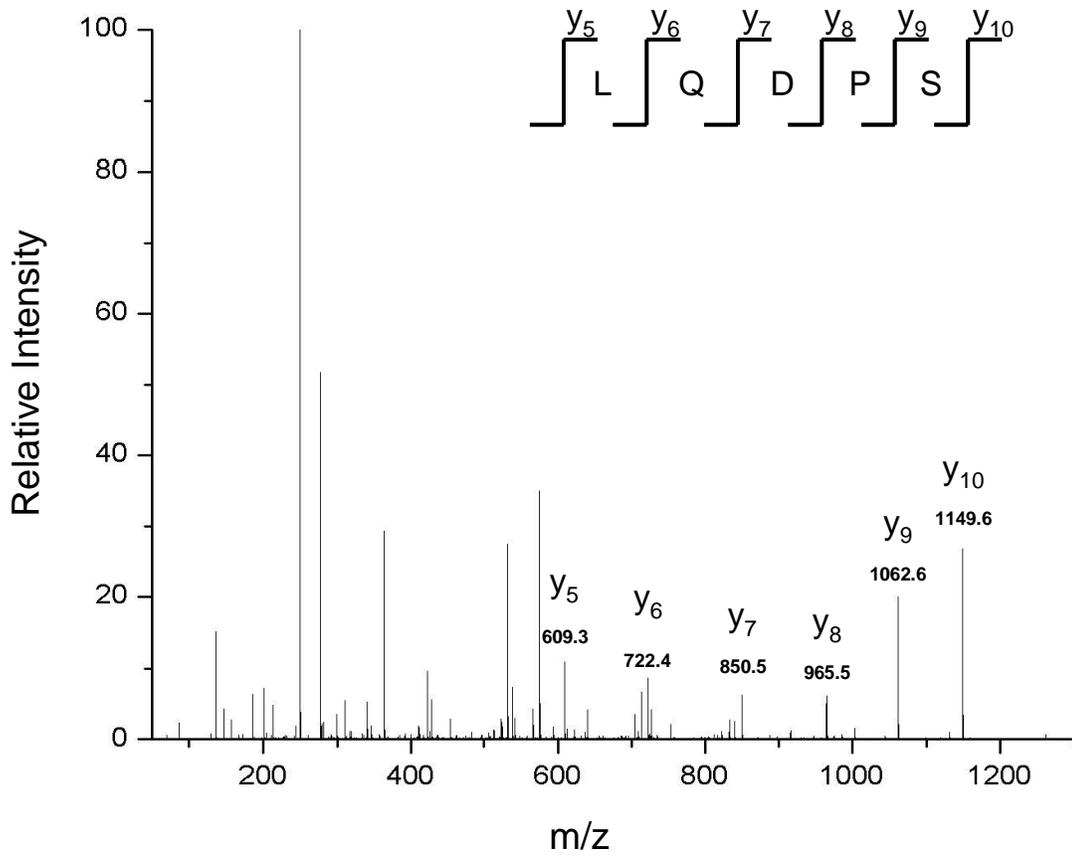
Figure 5: Tandem mass spectrum of a doubly charged peptide with an m/z of 713.36.

Protein identification can be obtained using this sequence information in two ways. An amino acid sequence tag may be obtained by identifying 4 or more amino acids in the observed peptide that are in sequence. This information, along the mass of the parent ion, the mass of the peptide on the N-term and C-term sides of the sequence tag, and the enzyme used for cleavage is then entered into a protein database and a list of probable protein identifications is generated by comparison against known peptides from this protein database. The identifications are then given with individual scores and probabilities.[33] Protein identification can also be obtained by comparing the MS/MS fragmentation spectrum directly with theoretical MS/MS spectra generated for peptides in a protein database. Protein identifications are then generated with scores and probabilities as before.[33] For organisms with genomes which have been sequenced, this mass spectrometry data may be used to compare with theoretical proteins predicted from the DNA sequence information.

C. Protein/Peptide Separation and Enzymatic Digestion

As protein mixtures increase in complexity, the analysis of proteins in the sample becomes more difficult, and the likelihood of identifying or analyzing less abundant components of these mixtures decreases. One way to overcome this obstacle is to use some protein separation technique upstream from the mass spectrometry analysis. Separation in thismanner is based on the physical properties of individual proteins such as size, charge, or affinity.

Historically, one of the most common ways to separate proteins is to perform an SDS-PAGE analysis. Proteins are placed in a buffer containing 2% SDS. The SDS both denatures the proteins and associates tightly with them and therefore gives each protein a large negative charge. They are then loaded into wells on a polyacrylamide gel. The gel is placed in a buffer containing SDS, and an electric current is applied to the gel. Because all of the proteins have a net negative charge, the electric current will separate the proteins based on their size or Stokes radii.[34] By changing the percentage of the polyacrylamide in the gel, separations may be optimized to increase the resolution for proteins of either low, medium, or high molecular weights. A good way to look at all protein mass ranges without sacrificing the resolution of a particular mass range is to use a gel with a continuous or discontinuous polyacrylamide percentage gradient such as 4-15%.

There are some drawbacks to this technique. The resolution of proteins from complex mixtures is poor and only rudimentary separation is obtained. Proteins can

aggregate in the sample wells and subsequently not migrate into the gel. Membrane proteins sometimes smear due to incomplete or heterogeneous incorporation of SDS.[35; 36; 37] Having mentioned these limitations, there are also some advantages to this technique. The technique is well established and widely used. SDS-PAGE also has a high loading capacity. Although membrane proteins do not migrate in well behaved tight bands as their soluble counterparts do, separation of membrane proteins is still attained using this technique.

Another gel-based technique is 2 dimensional gel electrophoresis (2DGE). 2DGE separates proteins not only by their size but by their charge, or isoelectric point (pI). The pI of a protein is the pH at which the net charge of the protein is zero. The technique of separating proteins by their pI is called isoelectric focusing (IEF). Membrane proteins are poorly represented when separated by 2D gel electrophoresis.[38; 39; 40] This is predominantly due to their incompatibility with the IEF step. Membrane proteins require high concentrations of detergents to remain soluble in solution and detergents interfere with the IEF step. Membrane proteins also tend to precipitate at their pI and do not enter the 2nd dimension SDS-PAGE step.

Another way to separate mixtures of proteins and peptides is to use high performance liquid chromatography (HPLC). This is a non-gel based method that can separate proteins based on their size, charge, hydrophobicity or other physical properties. There are two phases in HPLC; a stationary phase, and a mobile phase. The stationary phase is made up of material that has an affinity for a particular physical or chemical

property of proteins. The mobile phase is made up of organic or aqueous solvents, which flows through the stationary phase. The mobile phase is changed during the course of the chromatographic experiment to increase or decrease the affinity of the sample for the stationary phase. Using this technique one can separate complex mixtures of proteins and peptides into pure components or smaller subsets thatcan be more easily analyzed. Some of the more common forms of chromatography used for the separation of proteins and peptides are size exclusion chromatography (SEC), which separates components based on their size, ion exchange chromatography (IEC), which separates components based on their charged state, and reverse phase chromatography (RPC), which separates components based on their polarity.

As stated earlier, the vast majority of proteomic analyses are done using the "bottom-up" approach. Therefore, the protein or proteins of interest must be cleaved with an enzyme or chemical in order to obtain peptides for the mass spectrometry analysis. One of the ways this is achieved is to first separate the proteins using some gel based method such as SDS-PAGE. Following protein separation, the protein or proteins are excised from the gel using a scalpel or a robot to segment the desired area of the gel. This can be a protein gel spot, such as one obtained from a 2D gel, or a protein gel band, such as the type obtained from an SDS-PAGE experiment. Because of the high resolution obtained from 2DGE, the protein gel spots usually contain only 1 protein. Unfortunately, due to the low resolving power of SDS-PAGE, protein gel bands obtained from SDS-PAGE gels rarely contain only 1 protein and may often contain more than 10 depending on the size of the band excised from the gel.

Following the excision of the protein from the gel, the excised gel piece is then washed several times with aqueous and organic solvents to remove any excess salts or detergents from the gel that would interfere with the digestion process. Following this, the gel piece is placed in a solution containing dithiothreitol to reduce disulfide bonds. Next the gel piece is placed in a solution of iodoacetamide to alkylate the reduced cysteines so they do not reform disulfide bonds. Following this, the gel piece is dehydrated, and rehydrated in a solution containing the proteolytic enzyme trypsin and allowed to incubate overnight at 37ºC. By performing this procedure, the trypsin can enter the gel piece, bind the protein of interest and cleave the protein while it is still embedded in the gel matrix. Peptides are recovered from the gel pieces using acidified water and organic solvents and the extracted peptides are then analyzed by mass spectrometry.

Another "bottom-up" strategy for the analysis of proteins using mass spectrometry is to digest the proteins in-solution before analysis by mass spectrometry. This can be done in two different ways. Intact proteins can be fractionated using one or more of the chromatographic techniques mentioned earlier. These fractions can then be digested in a buffered enzymatic solution and analyzed by mass spectrometry using one of the previously describes methods. Complex mixtures of proteins can also be digested without any pre-fractionation and the resulting peptides can then be separated using one or more of the previously described chromatographic techniques. Most commonly a 2 dimensional separation of a peptide mixture consists of an initial

separation by ion exchange chromatography. After that each of the fractions obtained is then separated by reverse phase chromatography and analyzed by mass spectrometry.[41] This technique is often called "shotgun" proteomics. By using this 2 dimensional separation technique, which is sometimes automated, complex mixtures of peptides can be resolved and many proteins identified, including some of the less abundant ones.

Multidimensional chromatographic separation steps and analysis using mass spectrometry can be performed separately or the liquid chromatography steps can be coupled directly to a mass spectrometer (LC-MS). The peptides or proteins elute directly from the liquid chromatography instrument into the mass spectrometer for analysis and sophisticated software is used to analyze the samples in real time. This type of analysis can be done both with proteins digested in-solution or with proteins that have been previously separated by any gel-based method and subjected to in-gel digestion to recover the peptides. Using LC-MS to analyze peptides recovered from SDS-PAGE gels can allow the characterization of hundreds of proteins from a single gel, making this a very powerful tool for analysis.

Chapter 2: Plasma Membrane Isolation Technique

A. Membrane Protein Overview

Biological membranes are made up of lipids and proteins. These lipids form a bilayer, with polar head groups associated with the aqueous cellular environments and non-polar tail groups associated with each other creating a hydrophobic environment within the membrane. There are different types of membrane proteins associated with biological membranes. **Figure 6** shows some of these: [42]

- Single pass transmembrane proteins – proteins which pass from one side of the membrane to the other once.

- Multiple pass transmembrane proteins – proteins which pass from one side of the membrane to the other more than once.

- Lipid linked membrane proteins – proteins which have lipids covalently linked to them which anchor the protein to the membrane.

- Peripheral membrane proteins – Proteins which are associated with the membrane or other proteins in the membrane but are not inserted into it or covalently bound to it.

Membrane proteins can also be combinations of these types, such as a protein which is both lipid linked and transmembrane spanning, or proteins partially inserted into the membrane without passing completely through to the other side.

Biological membranes are fluid, and membrane proteins are free to move about the membrane.[42] Membranes act as a two dimensional solvent for membrane proteins the way the cytoplasm acts as a three dimensional solvent for soluble proteins. In prokaryotes, the only membrane is the plasma membrane so they contain no internal membrane organelles. Eukaryotes, on the other hand, contain internal organelles with membranes. These internal membranes serve to compartmentalize these structures in the cell, which have their own functions.

This dissertation outlines a method for the identification and characterization of proteins found in the plasma membranes of cell cultures. The first step in this analysis is the subcellular fractionation of a plasma membrane fraction. As is stated in the previous chapter, the plasma membrane makes up only a small percentage of the cell and its isolation can be difficult. In eukaryotes there are internal membranes present in much higher abundances than the plasma membrane and these need to be separated.

Subcellular fractionation before proteomic analysis can greatly increase the number of protein identifications by simplifying the complexity of the sample used for analysis. The cytosol is the easiest of these fractions to isolate and also the most abundant.[18] Other organelles such as the nuclei and mitochondria are easily isolated because they comprise individual units, each of a particular size and enclosed in
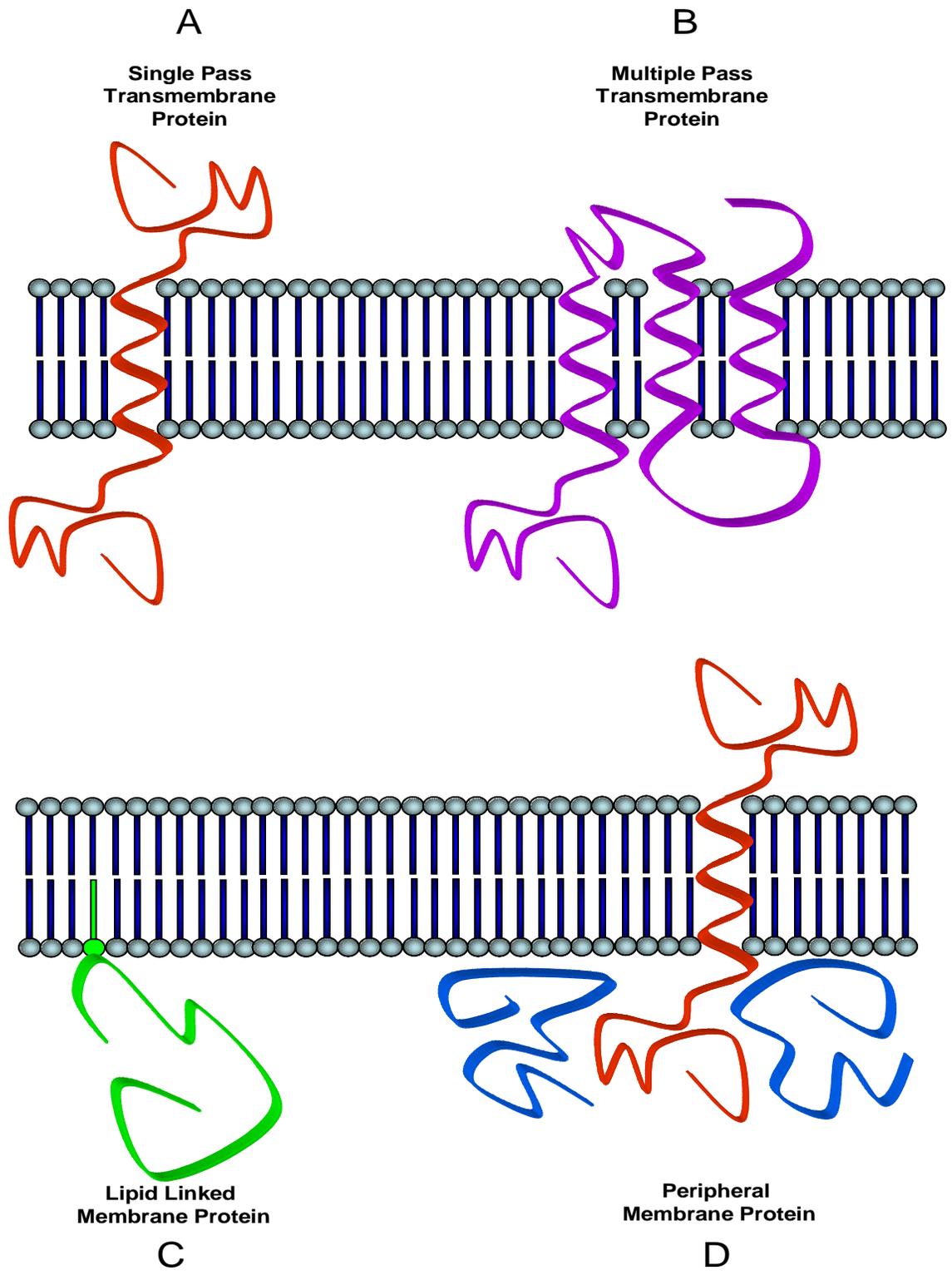
Figure 6: Different types of membrane proteins

their own membrane and they are readily separated by centrifugation.[43] Other organelles such as the endoplasmic reticulum, the Golgi apparatus, and the plasma membrane are more amorphous and harder to separate. Some membrane isolation techniques recover all internal membranes together after cell lysis.[44; 45] Since the total membrane fraction constitutes a large portion of the cell volume, and the plasma membrane makes up only a small portion of that, isolation of total membranes will produce significant contamination of the plasma membrane by proteins from other membranes.[18]

The most common ways to isolate these internal membrane structures separately is to use density gradient centrifugation using some non-ionic gradient material such as sucrose.[43] The problem with this method is that the plasma membrane does not always remain intact. It can take the form of open sheets and vesicles of different sizes and may be present in many different gradient fractions recovered during the isolation procedure.[43]

With the objective of obtaining a good plasma membrane sample for proteomic analysis, we have evaluated a rarely used literature technique for the isolation of plasma membranes, in which a pellicle is formed from cationic colloidal silica.[46; 47] This technique uses a suspension of positively charged silica micro beads to coat the exterior of cells in culture. These silica beads are electrostatically attracted to the exterior of the plasma membranes.[46] Once the cells have been covered with silica beads, the pellicle is stabilized with long polymers of acrylic acid. This also shields the positive charges exposed on the surface of the silica beads. After cells

are coated in this manner, they can be lysed and the plasma membranes are easily isolated from the rest of the internal membranes because of the increase in their density facilitated by the silica. We have modified the published method by addition of washing steps after the plasma membranes have been recovered by centrifugation.[43; 48]

This technique works with both suspension cells and cells which require a surface or solid support for growth. For cells grown on surfaces, the apical and basolateral plasma membrane domains can be isolated separately. By coating the cells still adhered to the cell culture flask only the apical plasma membrane is exposed to the coating solution and coated. The cells are then lysed while still attached to the flasks. Once the cell lysate is removed, the basolateral domains remain attached to the cell culture flask, facilitated by the structural proteins which anchor the cells to the flask during growth. This can then be cleaned up with a series of stringent washes, scraped off of the culture flask, and isolated by centrifugation.[48]

B. Materials and Methods

Materials

Criterion precast gels (13.3 x 8.7 cm, 12.5%, 8-16%, and 4-15%), Biosafe Coomassie stain, 10x PBS, 10x Tris/Glycine/SDS Buffer, 10x Tris/Glycine Buffer, pre-stained protein broad range standards, Laemmli sample buffer, nitrocellulose, and filter paper were purchased from Bio-Rad (Hercules, CA) . $MgCl_2$, MES, NaCl, LUDOX-CL cationic colloidal silica, polyacrylic acid (100,000 typical molecular weight), imidazole, Nycodenz, Na_2CO_3, EDTA disodium salt, protease inhibitor cocktail, ProteoQwest Colorimetric Western Blotting Kit, mouse α-human Na/K ATPase primary antibody, DTT, fetal calf serum, and Penicillin-Streptomycin solution were purchased from Sigma Aldrich (St. Louis, MO). Improved Minimal Essential Medium was purchased from American Type Culture Collection (Manassas, VA). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA).

Equipment

The Criterion precast gel system, Mini-PROTEAN 3 electrophoresis system, Mini trans-blot cell, and GS-800 densitometer were purchased from Bio-Rad (Hercules, CA).

Cell Culture

The human breast cancer mitoxantrone resistant cell line MCF-7 (MXR MCF-7) was a gift from Dr. K. H. Cowan (The Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska) and the human multiple myeloma cell line RPMI 8226 was a gift from Dr. R. Fenton (The Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore Maryland). MXR MCF-7 cells were grown in 150 cm^2 cell culture flasks using Improved Minimal Essential Medium containing 5% fetal calf serum and antibiotics at 37°C and 5% CO_2.[49] RPMI 8226 cells were grown in the same fashion but using RPMI 1640 media and heat inactivated fetal calf serum.[50]

Preparation of the Plasma Membrane Fraction from Suspension Cell Cultures

Plasma membranes from RPMI 8226 were isolated using a modification of the cationic colloidal silica plasma membrane isolation procedure used for suspension cells.[43; 46] About 1.5g wet weight RPMI 8226 cells were washed in plasma membrane coating buffer A (PMCBA – 20 mM MES, 150 mM NaCl, 800 mM sorbitol, pH 5.3) and placed drop-wise using a syringe and needle into a 10% suspension of cationic colloidal silica in PMCBA. The suspension was rocked gently on ice for 15 minutes. The silica-coated cells were then sedimented at 900g for 5 minutes to remove them from the silica solution.

The silica-coated cells were then washed with PMCBA and sedimented once more at 900g for 5 minutes to remove any excess silica. The cells were then placed drop-wise, using a syringe and needle, into a solution of 10mg/ml polyacrylic acid in PMCBA pH 6-6.5 and placed on ice for 15 min with gentle rocking (**Figure 7**).
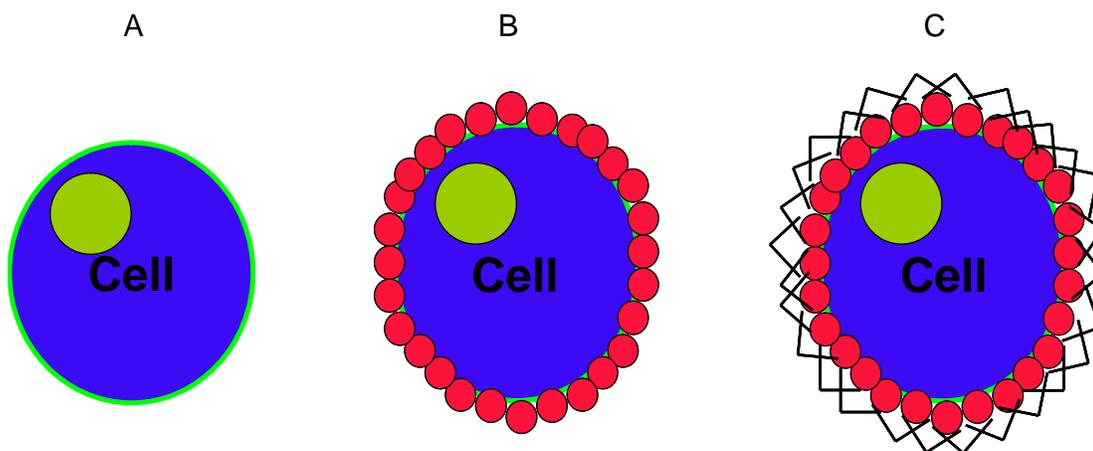
Figure 7. Scheme of the coating process using suspension cells. A) Uncoated suspension cell. B) Suspension cell coated with cationic silica. C) Suspension cells coated with cationic silica, followed by polyacrylic acid.

The cells were then washed with PMCBA, and placed in lysis buffer with protease inhibitors (2.5mM imidazole with Sigma protease inhibitor cocktail) and left on ice for 30 minutes to swell the cells. Cells were then lysed using nitrogen cavitation at 1500 psi. The cell lysate was spun at 900g for 30 min to sediment nuclei and silica coated plasma membranes. The pellet was resuspended in lysis buffer, diluted with an equal amount of 100% Nycodenz to make a 50% Nycodenz solution and layered over 70% Nycodenz. Lysis buffer was then layered onto each tube and filled to the top. The tubes were spun at 60,000g in an SW60Ti rotor for 23 min. The silica coated plasma membranes pellet to the bottom of the tube leaving the nuclei at the 50%/70% Nycodenz interface (**Figure 8**).

The supernatant was drawn off and the silica coated plasma membrane pellets were resuspended in lysis buffer (2.5 mM imidazole pH 7). The pellet was resuspended in lysis buffer and centrifuged at top speed on a bench top microfuge to remove the excess Nycodenz . The pellet was washed in this same way 2 additional times with lysis buffer and 3 times with 100 mM Na_2CO_3 pH 11.4, for a total of 6 washes. The purified plasma membrane proteins were then recovered from their silica coating by solubilization directly in Laemmli loading buffer (Bio-Rad), incubation in a 60ºC water bath for 30 min, sonication 5 times for 10 seconds at max setting, and incubation in a 60ºC water bath for an additional 30 minutes. The suspension was then spun at max speed in a bench top centrifuge for 15 min to pellet the silica coating from the solubilized plasma membrane proteins. The supernatant, which now

Cell lysis   Low g Spin   High g Spin Over
                                                         Nycodenz

Nuclei ──────────▶

Nuclei and Plasma   Purified Plasma
Membrane   Membrane
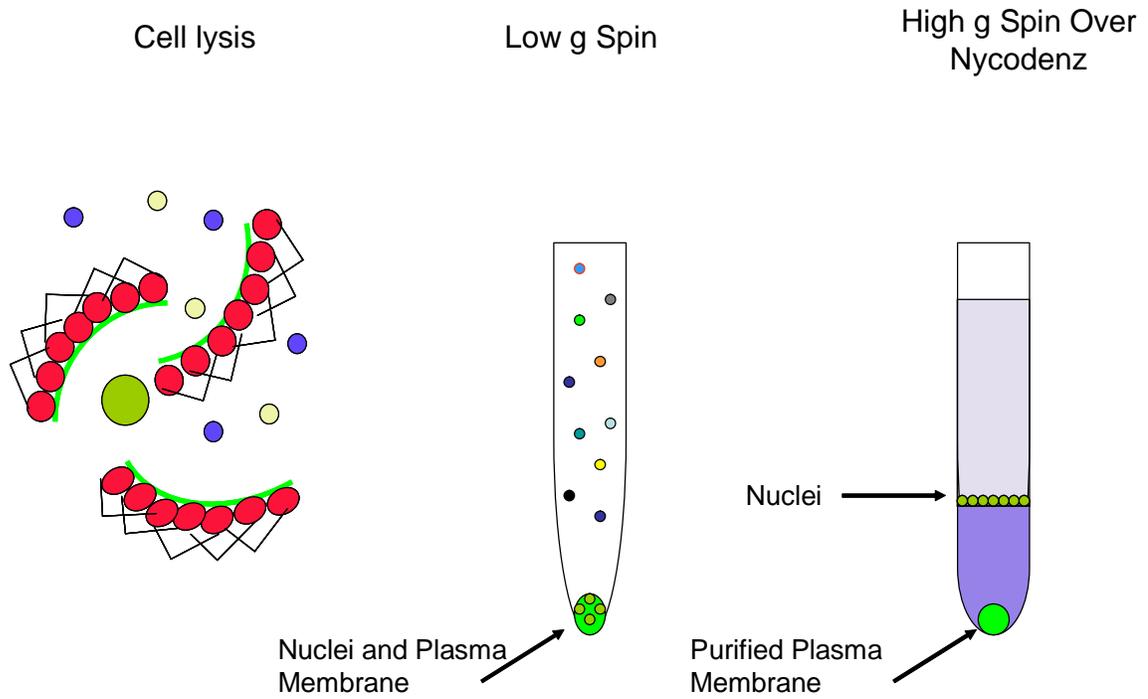
Figure 8. Scheme for the isolation of the coated plasma membranes from other
cellular organelles.

contained the solubilized plasma membrane proteins in the Laemmli buffer, was drawn off and stored at -80ºC and the pellet was discarded.

Preparation of the Plasma Membrane Fraction from Cell Culture Monolayers

Plasma membranes from MXR MCF-7 cells growing as monolayers were isolated using another modified cationic colloidal silica plasma membrane isolation procedure.[43; 47] In this case the cells were coated with the silica and polyacrylic acid while still attached to the cell culture flasks, so that all washes and cell coating solutions were added directly to the flasks with the cells still attached and decanted from the flasks to remove. MXR MCF-7 cells were grown to confluence in 150 cm^2 cell culture flasks. The media was removed and the cells were washed twice with PBS containing 1 mM $MgCl_2$, and 1 mM $CaCl_2$ and then washed with plasma membrane coating buffer B (PMCBB - 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM MES, 135 mM NaCl, pH 5.3). The cells were then coated with a 5% suspension of cationic colloidal silica in PMCBB and left on ice or on a metal surface in the cold room for 1 minute. The silica suspension was removed, followed by a wash with PMCBB to remove excess silica. The cells were then coated with a 10mg/ml solution of polyacrylic acid in PMCBB pH 6-6.5 and left on ice or on a metal surface in the cold room for 1 minute. The polyacrylic acid solution was removed, followed by a wash with PMCBB to remove any excess polyacrylic acid. The cells were washed once quickly with lysis buffer (2.5 mM imidazole pH 7) and then lysis buffer with Sigma protease inhibitor cocktail was added to the cell culture flasks, which are left on ice or

in the cold room for 30 min to swell the cells. The flasks were then placed on a bench

top and allowed to reach room temp (~15-30 min). The apical part of the plasma

membranes (the part of the plasma membrane coated by the silica and polyacrylic

acid) was sheared from rest of the cell by pipetting the lysis buffer in each flask up

and down over the cells, or by using a syringe with a long needle to spray the lysis

buffer directly onto the cells in order to apply enough shearing force to rip off the top

part of the plasma membranes (**Figure 9**).

At this point the apical membranes were drawn off with the pipette-aid and were

treated as described in the previous section. Following the removal of the nuclei using

the Nycodenz gradient, the silica coated plasma membrane pellet was washed 3 times

with lysis buffer. The silica coated plasma membrane sheets were then resuspended in

30 ml of 100 mM Na_2CO_3 pH 11.4 in a 50 ml centrifuge tube and placed in a

sonication bath for 30 minutes with vortexing every 5 minutes. The silica coated

plasma membrane sheets were pelleted at 16000g in an SW28 rotor for 30 min. The

pellet was resuspended in 30 ml of 100 mM Na_2CO_3 pH 11.4 in a 50 ml centrifuge

tube, sonicated as before and pelleted again in the same fashion. The pellet was then

resuspended in 1.5 ml of Na_2CO_3 and spun at maximum speed for 30 min in a

tabletop microfuge. The apical plasma membrane pellet was solubilized in 2% SDS,

or directly in Laemmli loading buffer and the plasma membrane proteins were

solubilized using sonication and a 60°C water bath as described in the previous

section.

Cell Lysis

Wash away cell debris

Basolateral Membranes
Still Attached to Cell
Culture Flask
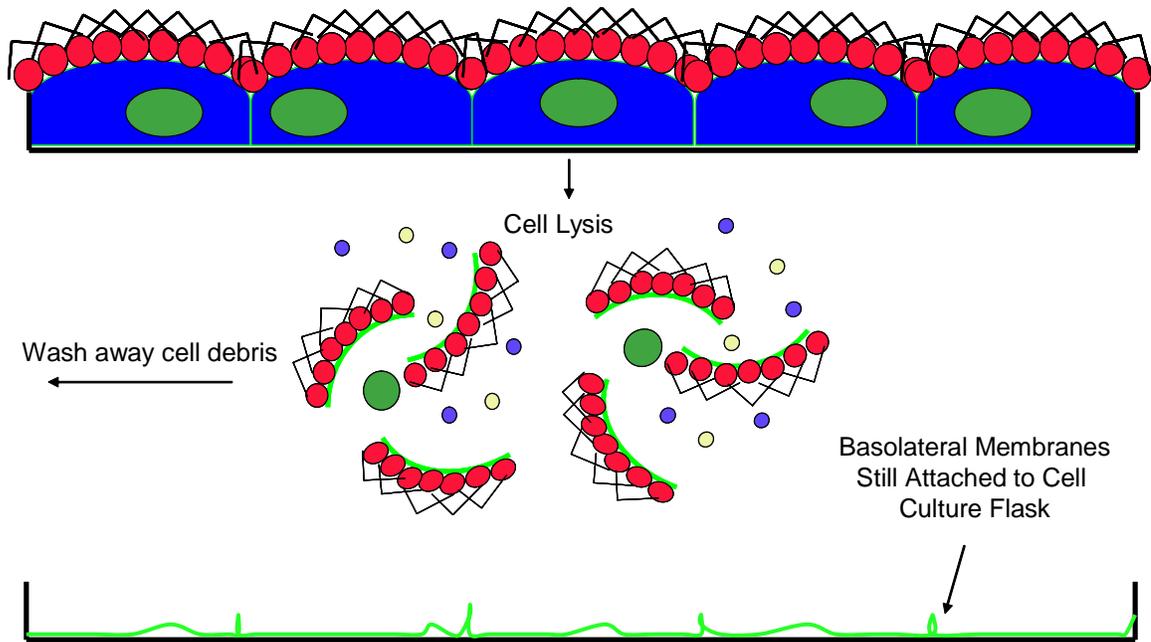
Figure 9. Scheme for the isolation of apical and basolateral plasma membranes from cells which require a solid surface for growth.

At this point the basolateral plasma membranes were still attached to the cell culture flasks. Following cell lysis, the cell culture flasks were washed once quickly with lysis buffer and then lysis buffer with protease inhibitors was added to each flask and left at 4ºC until the previous steps involving the isolation of the apical plasma membrane proteins was finished. The flasks were then removed from the cold room and incubated for 5 min at room temperature with rocking. The lysis buffer was poured off and 5M NaCl was added to each flask and incubated for 5 min at room temperature with rocking. The 5M NaCl was poured off and PBS containing 10 mM EDTA was added to each flask and incubated for 5 min at room temperature with rocking. The PBS/EDTA solution was poured off and washed once quickly with 100 mM Na_2CO_3, pH 11.4 to remove any excess PBS/EDTA. One hundred mM Na_2CO_3, pH 11.4 was added to each flask and incubated for 5 min at room temperature with rocking. The Na_2CO_3 was poured off and replaced with fresh 100 mM Na_2CO_3, pH 11.4 in each flask. The basolateral plasma membranes were then scraped from the bottom of the cell culture flask using a cell scraper and spun at 14,000g in an SW28 rotor for 20 min. The purified basolateral plasma membranes pellet to the bottom of the ultracentrifuge tubes and are then resuspended in a minimal amount of 100 mM Na_2CO_3 and transferred to a 1.5 ml microfuge tube and spun at max speed in a microfuge for 20 min to pellet the plasma membranes. The basolateral plasma membrane pellet can then be solubilized in 2% SDS or directly in Laemmli loading buffer and the plasma membrane proteins were further solubilized using sonication and a 60ºC water bath as described above.

One Dimensional SDS-PAGE and Western Blot Analysis

For Western analysis, 25 µg of protein was loaded onto 12.5% gels and run according
to specifications using the Bio-Rad Criterion precast gel system. Following
electrophoresis, the gels were either stained using Bio-Rad Biosafe Coomassie stain
for protein visualization or left unstained. Following electrophoresis, the proteins on
the unstained gels were transferred to a nitrocellulose membrane according to
specifications using the Bio-Rad Mini-PROTEAN 3 electrophoresis system and Mini
trans-blot cell. Western blotting was done according to specifications using the Sigma
ProteoQwest Colorimetric Western Blotting Kitand an antibody against the human
Na/K ATPase was used as the primary antibody. Following electrophoresis and
western blotting, the stained gels and nitrocellulose were scanned using the Bio-Rad
GS-800 densitometer. Enrichment of the plasma membrane fractions were quantitated
from the western blot images using the ImageQuant image analysis software by
Amersham Biosciences.

C. Results and Discussion

Western blot experiments were performed to determine the enrichment of the plasma membrane fractions isolated with this method using a mouse antibody against human Na+/K+ ATPase which is a commonly used plasma membrane marker.[43] The relative enrichments of each plasma membrane fraction were determined based on the densitometry measurement compared to that of the whole cell lysate. By this method, plasma membranes were determined to be enriched from RPMI 8226 suspension cells 18-fold (**Figure 10**), from the surface bound MXR MCF-7 cells by 8 fold and 20 fold for the apical and basolateral domains, respectively (**Figure 11**). **Table 2** shows the densitometry data obtained from the Image Quant program. The peak area from the densitometry data was used to determine plasma membrane enrichment.

The addition of the wash steps following the plasma membrane isolation increased the relative enrichment of plasma membranes when compared to the original method. The authors of the earlier paper describing the originally published method used a cell suspension of *Dictyostelium discoideum*, which is a soil living amoeba, and reported plasma membrane enrichment levels of 15-17 fold.[46] The present method reproducibly provides enrichment levels of 18 fold from human myeloma cells cultured in suspension.[48]

The enrichment of the basolateral plasma membrane was greatly increased by the present method. The enrichment for this domain was increased 20 fold after the
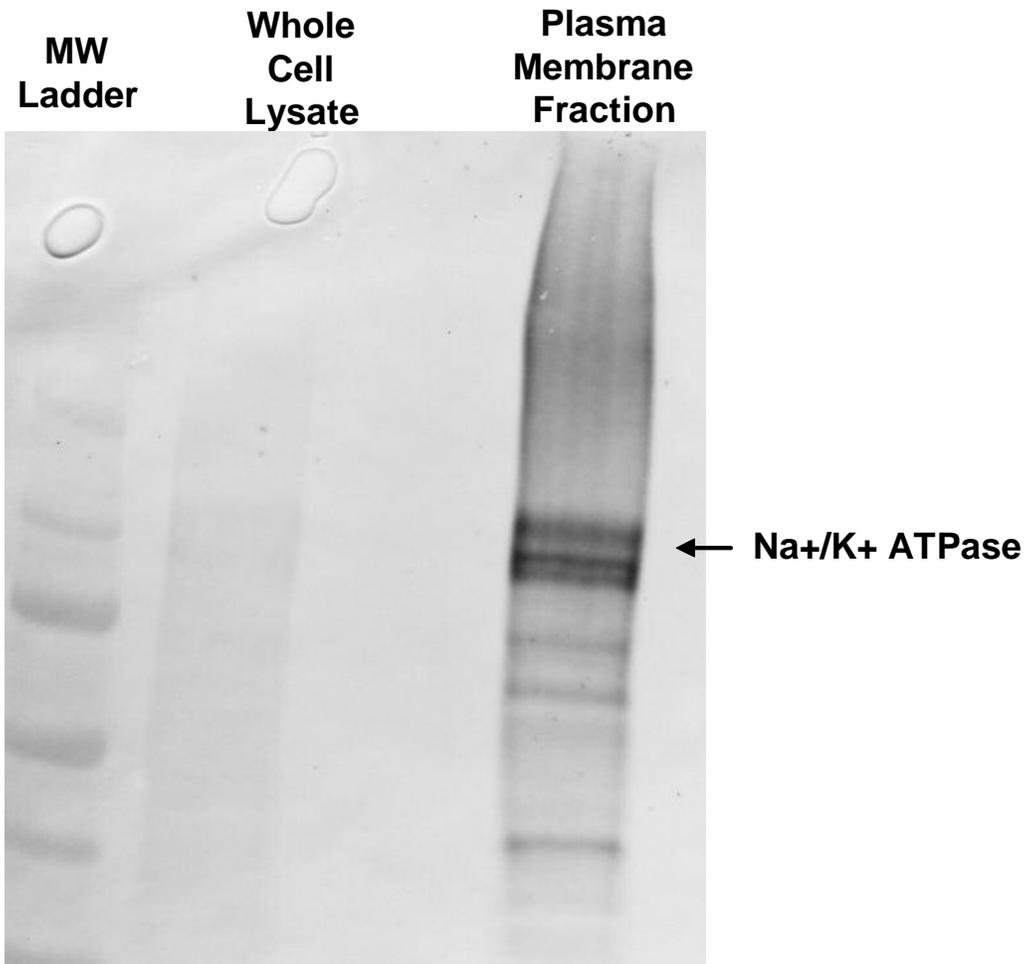
Figure 10: Western analysis of RPMI 8226 whole cell lysate and the isolated plasma membrane fraction using an antibody against the Na+/K+ ATPase to determine the plasma membrane enrichment.
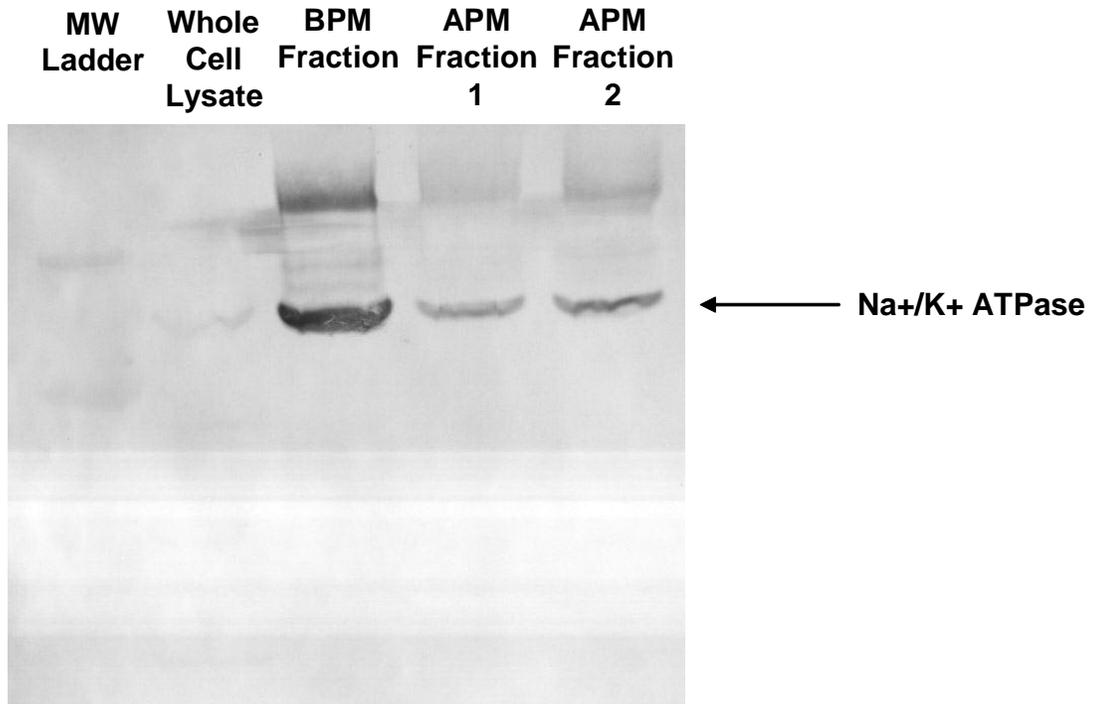
Figure 11: Western analysis of MCF7 whole cell lysate and the different isolated plasma membrane fractions using an antibody against the Na+/K+ ATPase to determine the plasma membrane enrichment.

A

Peak	Area	Height
Whole Cell Lysate	218	5
RPMI 8226 Plasma Membranes	4019	88

B

Peak	Area	Height
Whole Cell Lysate	138	7
Apical Plasma Membrane Fraction 2	1132	76
Basolateral Plasma Membranes	2750	115

Table 2: Densitometry data used to determine the enrichment of the isolated plasma membrane fractions relative to an unfractionated whole cell lysate. **A)** Data from the Multiple Myeloma cell line. **B)** Data from the MCF-7 cell line.

additional wash steps, a factor of 5 over the previously reported method. It has been

suggested that the basolateral domains, which are not covered with the silica and

polyacrylic acid, may not attract as much cytoplasmic contamination as their coated

apical counterparts do due to the electrostatic attraction to the charged silica and

polyacrylic acid.[46] In addition, the basolateral plasma membrane domains are also still

bound to the cell culture flasks after cell lysis. This enables a series of stringent

washes to remove most of the unbound cytoplasmic and membrane domain

contaminants without washing away the sample. After the contaminants are removed,

the isolated basolateral plasma membrane domains are recovered from the cell culture

flasks for further analysis.

Recently a novel method for the isolation of plasma membranes has been developed,

which uses a special reagent containing a biotin subunit linked to a functional group

that reacts with primary amine groups such as those in the amino acid side chains of

arginine and lysine.[51; 52] Using this technique, cell surface proteins are biotinylated

before cell lysis. After the cells have been lysed, the plasma membranes are isolated

using streptavidin immobilized on beads to capture the biotinylated proteins while

they are still embedded in the plasma membrane. Since this is done in an aqueous

environment, the membranes remain intact and the plasma membrane can be

isolated using the biotinylated proteins as anchors for the rest of the membrane.

This allows plasma membrane proteins, such as those which are only exposed to the

cytoplasmic face of the plasma membrane, to be isolated even if they were not

biotinylated as long as they are held firmly in the plasma membrane.

This method, termed Biotin Directed Affinity Purification (BDAP), achieved a comparable level of enrichment as our own, based on the analysis of the proteins identified.[51] However, there are some potential drawbacks. Our method for the isolation of plasma membranes yields both apical and basolateral domains of adherent cell cultures, while the BDAP method only isolates the apical plasma membrane domains. The BDAP method chemically modifies the lysine and arginine amino acid residues of the proteins tagged with this biotin reagent. Since these are the trypsin cleavage sites required for proteolytic digestion, there is the possibility that this chemical modification may inhibit proteolytic cleavage at these sites and reduce the amount of peptides recovered from tryptic digestions. Our method involves no covalent modification of the proteins themselves. Only electrostatic forces are used and the proteins are eventually recovered unaltered.

Although the silica method was designed for the isolation of plasma membranes from cell culture, it should also be effective using fresh tissue samples. After dissociating the tissue into individual cells using collagenases and other enzymes to cleave the connective proteins between the cells one can isolate their plasma membranes by performing the procedure as if they were suspension cell cultures.[43; 53] With recent advances in primary cell culturing techniques it is possible to isolate and culture primary cells directly from a patient and grow them on monolayers and therefore isolate the distinct apical and basolateral domains.[15; 43; 54]

Chapter 3: Proteomic Analysis of Plasma Membrane Proteins

A. Membrane Protein Analysis Overview

The objective of this portion of the experiment was to perform a "bottom-up" proteomic analysis on the purified plasma membrane protein samples isolated from cell cultures in the previous section. The mass spectrometry data collected in this experiment was then used for the identification of the proteins present in that sample. In order to achieve these results we had to devise a strategy for the digestion of the plasma membrane proteins isolated from this fraction into individual peptides that can subsequently be analyzed by mass spectrometry.

Although it is theoretically possible to do a" top-down" style of analysis for the analysis of proteins, that type of proteomic strategy has yet to be applied to the identification of membrane proteins.[38; 39] The reasons for this include the requirement for detergents to keep the membrane proteins in solution, which interferes with chromatographic separations and mass spectrometry ionization, and extensive and heterogeneous carbohydrate modifications found on membrane proteins which can significantly alter protein chemistry and diversify molecular masses used for identification.[55; 56]

There are not as many techniques available for the use in the analysis of membrane proteins as there are for soluble proteins. The "shotgun" approach to proteomic analysis has had only a minimal amount of success with the analysis of membrane proteins. This is primarily due to the fact that the solution digestion of membrane proteins is difficult because of the incompatibility of the conditions used for enzymatic digestions with the conditions necessary for the solubilization of membrane proteins.[57] 2DGE has obtained only limited success in the separation of membrane proteins. This is predominantly due to the inability of the IEF process to separate membrane proteins under the conditions used to keep membrane proteins in solution.[58]

Recently there has been a reappearance of the use of 1D SDS-PAGE for protein separation and analysis, and this has been especially true in relation to the separation of membrane proteins.[59; 60] Membrane proteins can be solubilized in SDS or Laemmli loading buffer and can subsequently be loaded directly onto SDS-PAGE gels.[48] Although the resolution of this technique is lower than 2DGE, it can provide a basic separation of proteins by molecular weight. Once separated, the gel bands can be excised from the gel using a razor and subjected to in-gel digestion using some digestive enzyme such as trypsin.[61] These peptides can then be analyzed by MALDI mass spectrometry, with or without LC separations, or electrospray mass spectrometry either by direct infusion or coupled to a liquid chromatography delivery system. By separating the peptides by liquid chromatography before introduction into the mass spectrometer, more peptides can

be analyzed from samples of complex peptide mixtures then without separation

using static infusion experiments.[48]

B. Materials and Methods

Materials

The Criterion precast gel system, Criterion precast gels (13.3 x 8.7 cm 4-15%),

Biosafe Coomassie stain, 10x Tris/Glycine/SDS Buffer, pre-stained protein broad

range standards, and Laemmli sample buffer were purchased from Bio-Rad (Hercules,

CA) . DTT, iodoacetamide, and TFA were purchased from Sigma Aldrich (St. Louis,

MO). NH_4HCO_3, formic acid, acetonitrile, acetic acid, methanol, and $CaCl_2$ were

purchased from Fisher (Pittsburg, PA). C18 ZipTips were purchased from Millipore

(Billerica, MA). Modified porcine trypsin was purchased from Promega (Madison,

WI).

Equipment

Mass spectra were obtained using an Applied Biosystems Qstar Pulsar i (Foster City,

CA) with a nanospray ion source from Protana (Odense, Denmark). NanoLC-MS was

performed online with the LC Packings Ultimate Nano LC System (Sunnyvale, CA).

NanoES Spray capillaries and an LC-MS head with Liquid Junction were purchased

from Proxeon (Odense, Denmark). Nanospray emitters were purchased from New

Objectives (Woburn, MA).

One Dimensional SDS-PAGE Analysis

Between 40 and 80 μg of plasma membrane protein was loaded onto 4-15% gels and run according to manufacturer's specifications using the Bio-Rad Criterion precast gel system. Following electrophoresis, the gels were stained using Bio-Rad Biosafe Coomassie stain and the stained gels were scanned using a GS-800 densitometer from Bio-Rad (Hercules, CA) .

Mass Spectrometry

The SDS-PAGE gel was excised into 28 gel bands and tryptic digestion was performed on the gel slices.[61] After extraction from the gel bands, the tryptic peptides were desalted using ZipTip C18 pipette tips. The acetonitrile/TFA was removed using a speed vac and the peptides were resuspended in electrospray ionization solution (methanol/water/acetic acid - 50/50/2) in preparation for static infusion nanospray MS/MS analysis, or placed in 0.1% formic acid (FA) in preparation for nanoLC-MS/MS analysis. The tryptic peptides were analyzed by static infusion using NanoES spray capillaries, and by online nanoLC-MS/MS. Reverse phase conditions were A: 97.5% H_2O/2.5% ACN/0.1%FA, B: 97.5% ACN/2.5% H_2O/0.1%FA with a 60 minute gradient from 5%-35%B on a PepMap 75μm I.D., 15cm, 3μm, 100Å column from LC Packings (Sunnyvale, CA).

Protein Identification

Each protein was identified based on sequences from two or more peptides using the integrated Qstar software Analyst QS with Bioanalyst and ProID for the LC-MS experiments, and the MASCOT search engine from Matrix Science for the offline static infusion nanospray experiments.[33] The peptides from the static infusion experiments were identified with a minimum confidence level of 95%. The peptides from the LC-MS experiments were identified with a minimum confidence level of 99.4% and were also manually sequenced from the tandem mass spectrometry data. **Figure 12** shows a scheme of the strategy used for the identification of proteins from the plasma membrane fractions.

1D SDS-PAGE
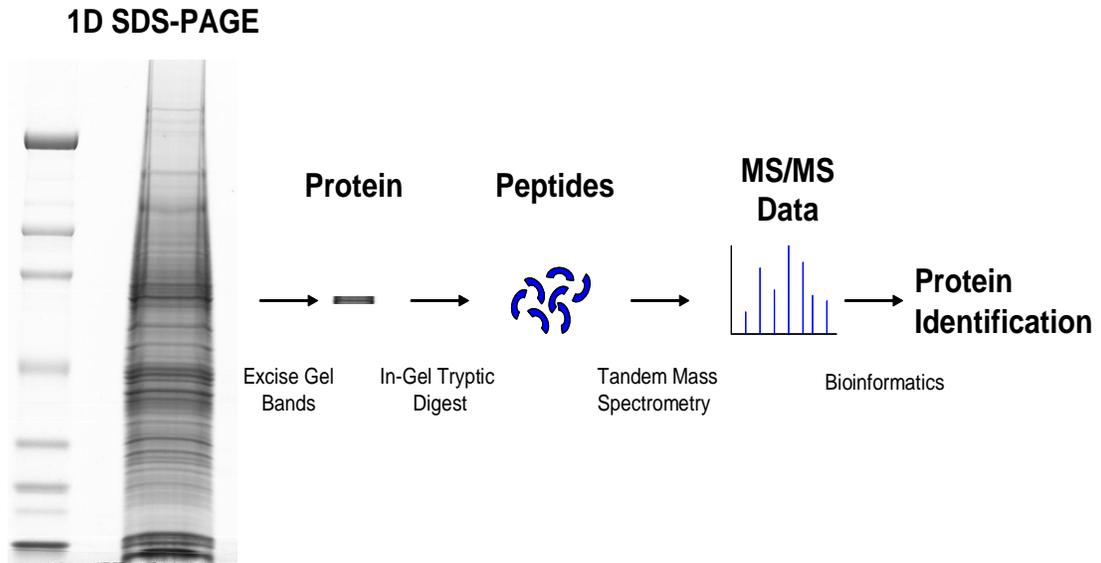
Figure 12: Strategy for the identification of proteins isolated from the plasma membranes of cell cultures.

C. Results and Discussion

Proteins Identified from RPMI 8226 Multiple Myeloma Cells using Static Infusion nanospray Mass Spectrometry

Seventy five µg of plasma membrane proteins isolated from the multiple myeloma cell line was loaded onto 4-15% polyacrylamide gels and subjected to SDS-PAGE (**Figure 13**). After protein separation, the gels were stained with Coomassie blue stain and cut horizontally into slices 3 mm wide. A total of 28 slices were excised from the gel and subjected to in-gel tryptic digestion. The peptides were recovered from the gel bands and placed in nanospray ionization solution and analyzed by electrospray ionization mass spectrometry without further separation.

Figure 14 shows mass spectra obtained from the analysis of the peptides recovered from band 14. **Figure 14 A** shows a survey scan of the total peptide mixture observed from this experiment. When analyzing a complex mixture of peptides without any type of separation it is hard to differentiate them from one another. Even in the inset it is hard to pick out the doubly charged peptide with a m/z of 639.3. However when this peptide is selected for tandem mass spectrometry it gives excellent fragmentation data and clear sequence tag of "FSDL" can be obtained (**Figure 14 B**). The peptide was found to originate from the neutral amino acid transporter B(0) protein (sp|Q15758).

Plasma Membrane Proteins   Whole Cell Lysate   MW(kDa)

75µg   50µg   25µg   12.5µg   25µg

250

150

100

75

50

37

25

20

15

1   2   3   4   5   6
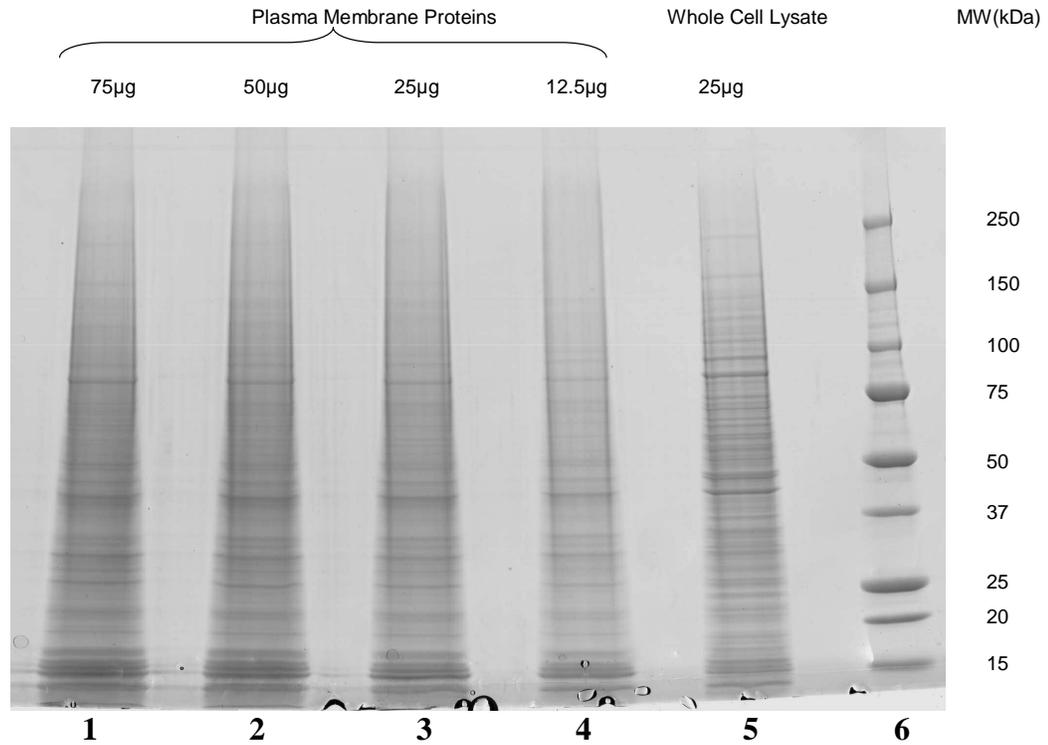
Figure 13: SDS-PAGE gel of proteins isolated from RPMI 8226 cell cultures. Lanes 1-4 contain 75 µg - 12.5 µg of plasma membrane proteins isolated using the previously described isolation method for suspension cell cultures. The lane containing 75 µg of protein was used for protein identification experiments. Lane 5 is 25 µg of total proteins isolated from RPMI 8226 without any fractionation, and lane 6 is molecular weight standards.

Figure 14: A. Survey scan of band 14 excised from an SDS-PAGE gel containing 75 µg of plasma membrane proteins from RPMI 8226 cells. The inset is an expanded view of the 639.3 doubly charged peptide. B. MS/MS scan of the 639.3 doubly charged peptide selected for tandem mass spectrometry with the y-ions used to obtain the sequence tag labeled above each peak.

Each of the 28 gel bands excised from the gel was analyzed in the same manner as described above. A total of 47 unique proteins were identified from the 28 gel bands with a minimum of 2 peptides identified from each protein. **Table 3** shows a list of the proteins identified from these experiments that have been previously characterized as plasma membrane proteins in the literature.

In the previous chapter we showed by Western blot analysis that the plasma membranes from RPMI 8226 cell cultures were enriched by 18 fold when compared to the abundance levels in the unfractionated whole cell lysate. Here we show that 20 of the 47 proteins identified from this fraction were identified as plasma membrane proteins corresponding to 43% (**Figure 15**).

Proteins Identified from MXR MCF-7 Breast Cancer Cells using Static Infusion nanospray Mass Spectrometry

Eighty μg of plasma membrane proteins isolated from the basolateral plasma membranes of MXR MCF-7 cell line was loaded onto 4-15% polyacrylamide gels and subjected to SDS-PAGE and analyzed in the same manner as the myeloma cell line (**Figure 16**). The basolateral plasma membrane fraction was used because the enrichment levels were much higher than the apical plasma membrane fraction. **Figure 17** shows mass spectra obtained from the analysis of the peptides recovered

Accession #	Protein Name
sp|P05023	Sodium/potassium-transporting ATPase alpha-1 chain precursor
sp|P08195	4F2 cell-surface antigen heavy chain
sp|Q15758	Neutral amino acid transporter B(0)
sp|P43007	Neutral amino acid transporter A (SATT)
sp|Q01650	Large neutral amino acids transporter small subunit 1
sp|Q99808	Equilibrative nucleoside transporter 1
sp|Q14242	P-selectin glycoprotein ligand 1 precursor
sp|P01891	HLA class I histocompatibility antigen, A-68 alpha chain precursor
sp|P04898	Guanine nucleotide-binding protein G(i), (Adenylate cyclase-inhibiting G alpha protein)
sp|P30685	HLA class I histocompatibility antigen, B-35 B*3505 alpha chain precursor
sp|P18827	Syndecan-1 precursor
sp|P54709	Sodium/potassium-transporting ATPase beta-3 chain
sp|P27105	Erythrocyte band 7 integral membrane protein (Stomatin)
sp|P13761	HLA class II histocompatibility antigen, DR-7 beta chain precursor
sp|P35613	Basigin precursor
sp|P01903	HLA class II histocompatibility antigen, DR alpha chain precursor
sp|P25507	Ras-related protein Rab-8
sp|P01911	HLA class II histocompatibility antigen, DW2.2/DR2.2 beta chain
sp|O88386	Ras-related protein Rab-10
sp|Q04941	Intestinal membrane A4 protein

Table 3: Plasma membrane proteins identified from RPMI 8226 multiple myeloma cells using offline static infusion nanospray mass spectrometry.

Plasma Membrane Proteins, 20, 43%

All Other Proteins, 27, 57%

Figure 15: Pie chart representing the amount and percentage of plasma membrane proteins identified in comparison to the total number of proteins positively identified from the plasma membrane fraction isolated from RPMI 8226 cells.

from band 19. A survey scan of the peptides recovered from band 19 is shown in **Figure 17 A.** The doubly charged peptide with a m/z of 626.7 was manually picked for analysis by tandem mass spectrometry and the spectrum is shown in **Figure 17 B**. The amino acid sequence tag "SDFD" can be determined from the fragmented y-ions observed. This peptide was found to be from the plasma membrane protein 4F2 cell surface antigen (sp|P08195).

A list of the proteins identified from the basolateral plasma membrane fraction from the MXR MCF-7 cell cultures is shown in **Table 4**. Out of a total of 40 proteins identified from this fraction, 20 were found to be plasma membrane proteins (**Figure 18**). Although half of the proteins identified were not localized to the plasma membrane, this method for the isolation and analysis of plasma membrane proteins for both suspension cells and adherent cells worked well enough to identify a significant percentage of proteins from the plasma membrane, a subcellular fraction which makes up only a tiny portion of the total cell content.

Proteins Identified from Mitoxantrone Resistant MCF-7 Breast Cancer Cells using nanoLC-MS

Our objective for this portion of the experiments was to identify as many proteins as possible that were present in the basolateral plasma membrane fraction isolated from MXR MCF-7 cells. Inspection of the SDS-PAGE gel experiment used in the static nanospray experiments (**Figure 16**), indicates more than 42 proteins present in the sample. A strategy was developed using a liquid chromatography delivery
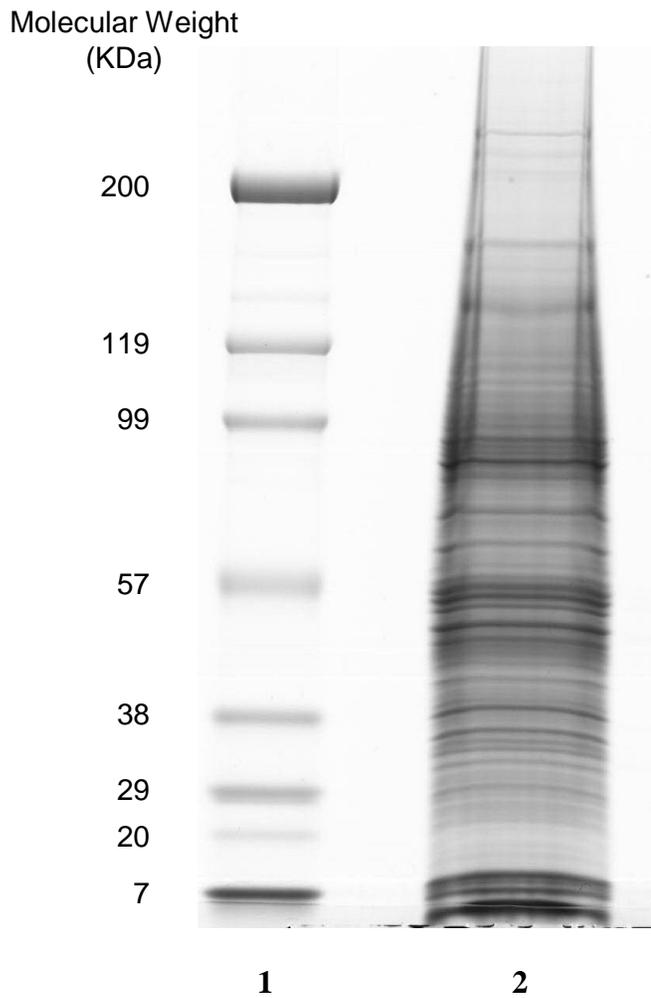
Figure 16: SDS-PAGE gel of proteins isolated from basolateral plasma membranes from MXR MCF-7 cell cultures. Lane 1, molecular weight standards. Lane 2, 80 µg of plasma membrane proteins isolated using the previously described isolation method for adherent cell cultures.
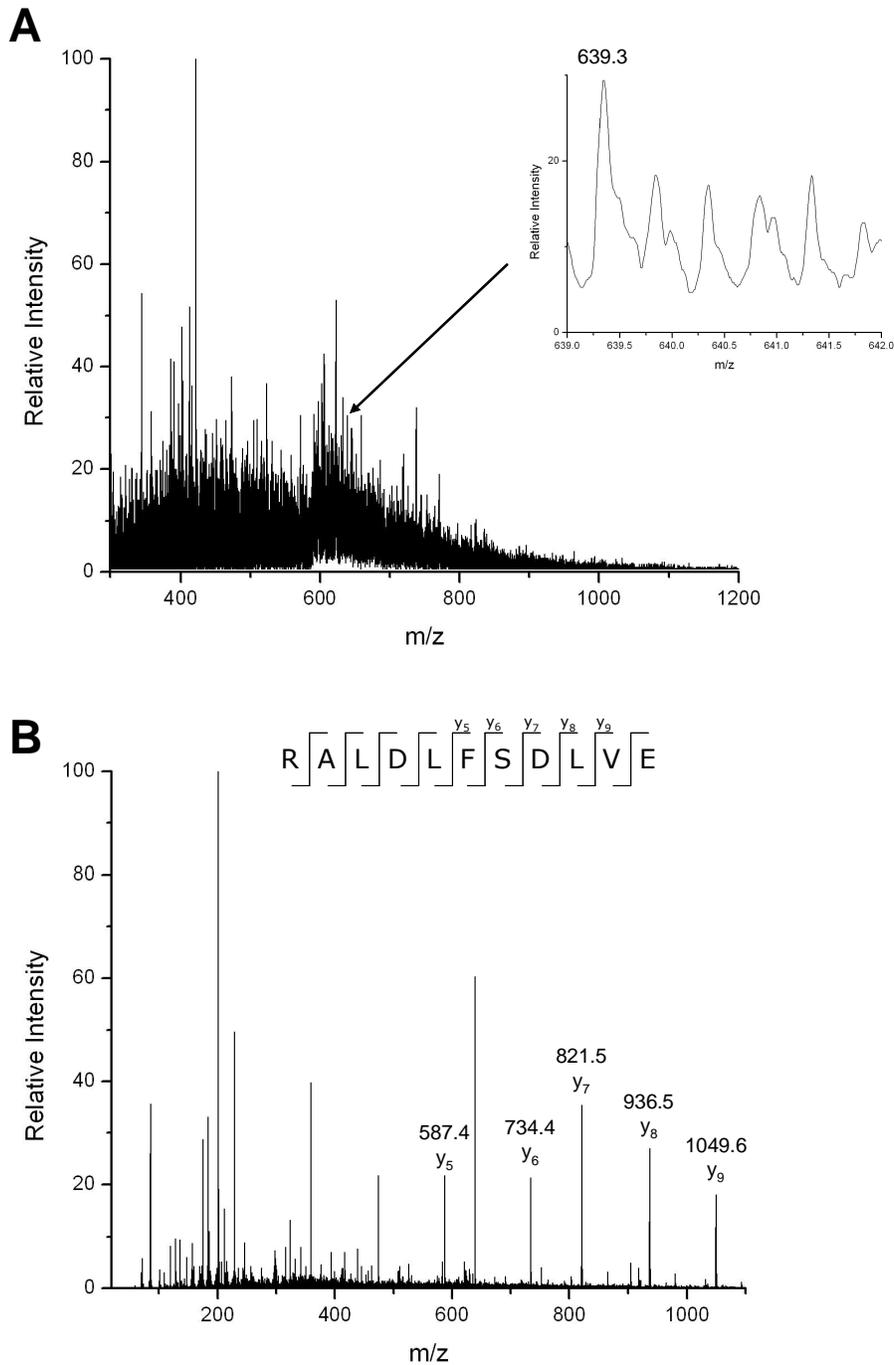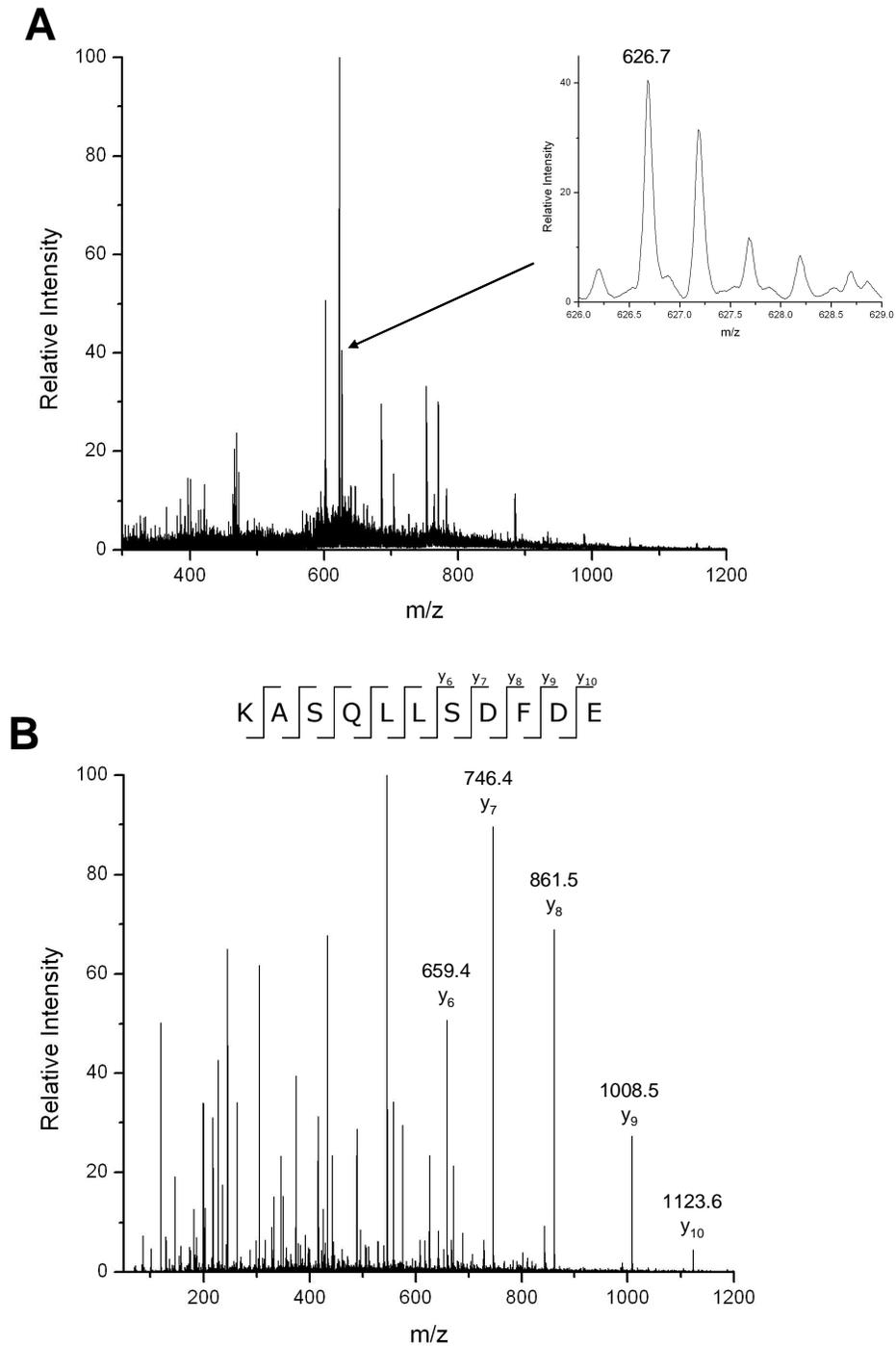
Figure 17: A. Survey scan of band 19 excised from an SDS-PAGE gel containing 80 µg of plasma membrane proteins from MXR MCF-7 cells. The inset is an expanded view of the 626.7 doubly charged peptide. B. MS/MS scan of the 626.7 doubly charged peptide selected for tandem mass spectrometry with the y-ions used to obtain the sequence tag labeled above each peak.

Accession #	Protein
sp|P17301	Integrin alpha-2 precursor (Platelet membrane glycoprotein Ia
sp|P06756	Integrin alpha-V precursor (Vitronectin receptor alpha subunit)
sp|P05023	Sodium/potassium-transporting ATPase alpha-1 chain precursor
sp|P02786	Transferrin receptor protein 2
sp|Q9UNQ0	BCRP, ATP-binding cassette, sub-family G, member 2,
sp|P08195	4F2 cell-surface antigen heavy chain
sp|Q03113	Guanine nucleotide-binding protein, alpha-12 subunit (G alpha 12) - Homo sapiens (Human).
sp|P11016	Guanine nucleotide-binding protein G(I)
sp|Q01650	Large neutral amino acids transporter small subunit 3
sp|Q00325	Phosphate carrier protein
sp|P16422	Tumor-associated calcium signal transducer 1 precursor
sp|P21796	Voltage-dependent anion-selective channel protein 1 (VDAC-1)
sp|P60033	CD81 antigen (26 kDa cell surface protein TAPA-1)
sp|P21926	CD9 antigen (P24)
sp|O15551	Claudin-3 (Clostridium perfringens enterotoxin receptor 2)
sp|P51148	Ras-related protein Rab-5C
sp|P51149	Ras-related protein Rab-7
sp|Q15286	Ras-related protein Rab-36
sp|O88386	Ras-related protein Rab-10
sp|Q9H0U4	Ras-related protein Rab-1B
sp|P09526	Ras-related protein Rap-1b

Table 4: Plasma membrane proteins identified from the basolateral region of MXR MCF-7 breast cancer cells using offline static infusion nanospray mass spectrometry.
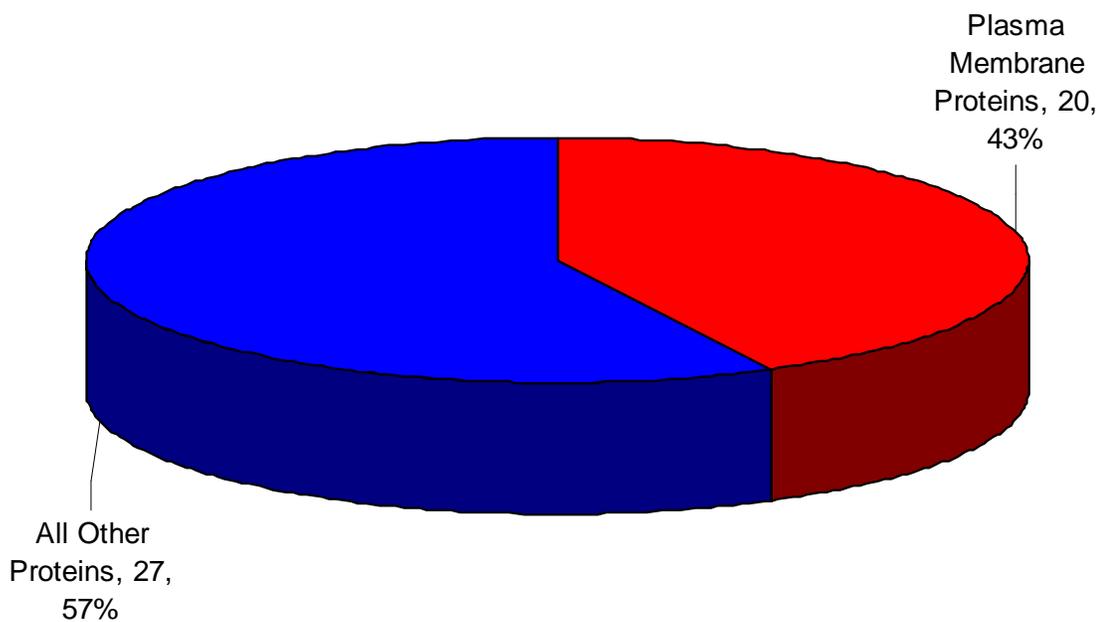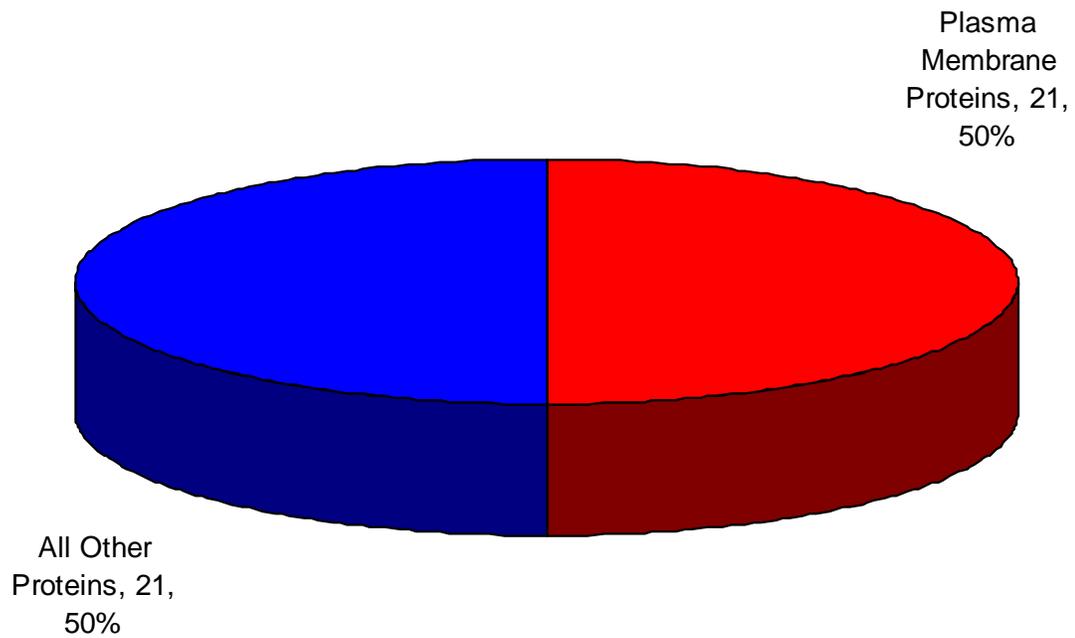
Figure 18: Pie chart representing the amount and percentage of plasma membrane proteins identified in comparison to the total number of proteins positively identified from the plasma membrane fraction isolated from MXR MCF-7 cells.

system coupled to a mass spectrometer to improve the number of protein identifications obtained and do so in a high throughput automated way.

Forty μg of plasma membrane proteins isolated from the basolateral plasma membranes of MXR MCF-7 cell line was loaded onto 4-15% polyacrylamide gels and subjected to SDS-PAGE (**Figure 19**). Protein gel bands were digested in-gel and the peptides were recovered as before. The proteins were then analyzed by nanoLC-MS in the information dependent acquisition mode.

Figure 20 shows the total ion current (TIC) chromatograms from the LC-MS analysis of bands 17 and 19. The gradient profile is from 5 to 35% solvent B over the course of 60 min. This allows each of the peptides to be scanned individually without interference from other ions of similar m/z values. The gradient does not begin until 10 min into the analysis and there is approximately 7 min of dead volume between the column and the mass spectrometer. The spike in the TIC at 17 min shows the switch in solvent flow from the desalting pre-column to the reverse phase nano-column and represents the beginning of the 60 min gradient (10 min of sample equilibration and desalting + 7 min of dead volume). The gradient is then finished at 77 min on the TIC. At 77 min, only a few of the extremely large hydrophobic peptides have yet to be released from the column and they eluted during the period of time after this where the solvent B concentration is ramped to 95 %. Most of the peptides have eluted from the column at 35% solvent B and it is not necessary to run the gradient to 50% solvent B or higher.
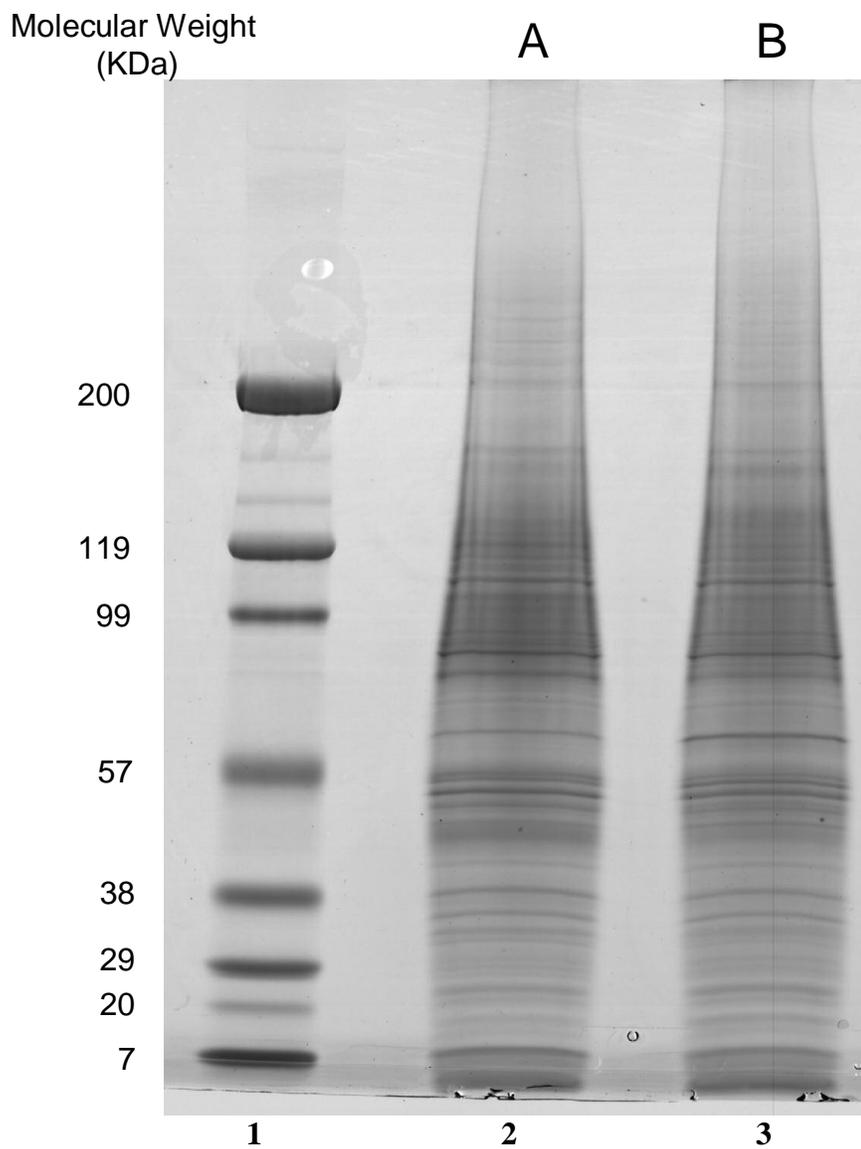
Figure 19: SDS-PAGE gel of proteins isolated from basolateral plasma membranes from MXR and drug susceptible MCF-7 cell cultures. Lane 1 – Molecular weight standards. Lane 2 – 40 µg plasma membrane proteins from MXR MCF-7 cells. Lane 3 – mixture of 20 µg plasma membrane proteins from MXR MCF-7 cells and 20 µg plasma membrane proteins from the drug susceptible MCF-7 cells
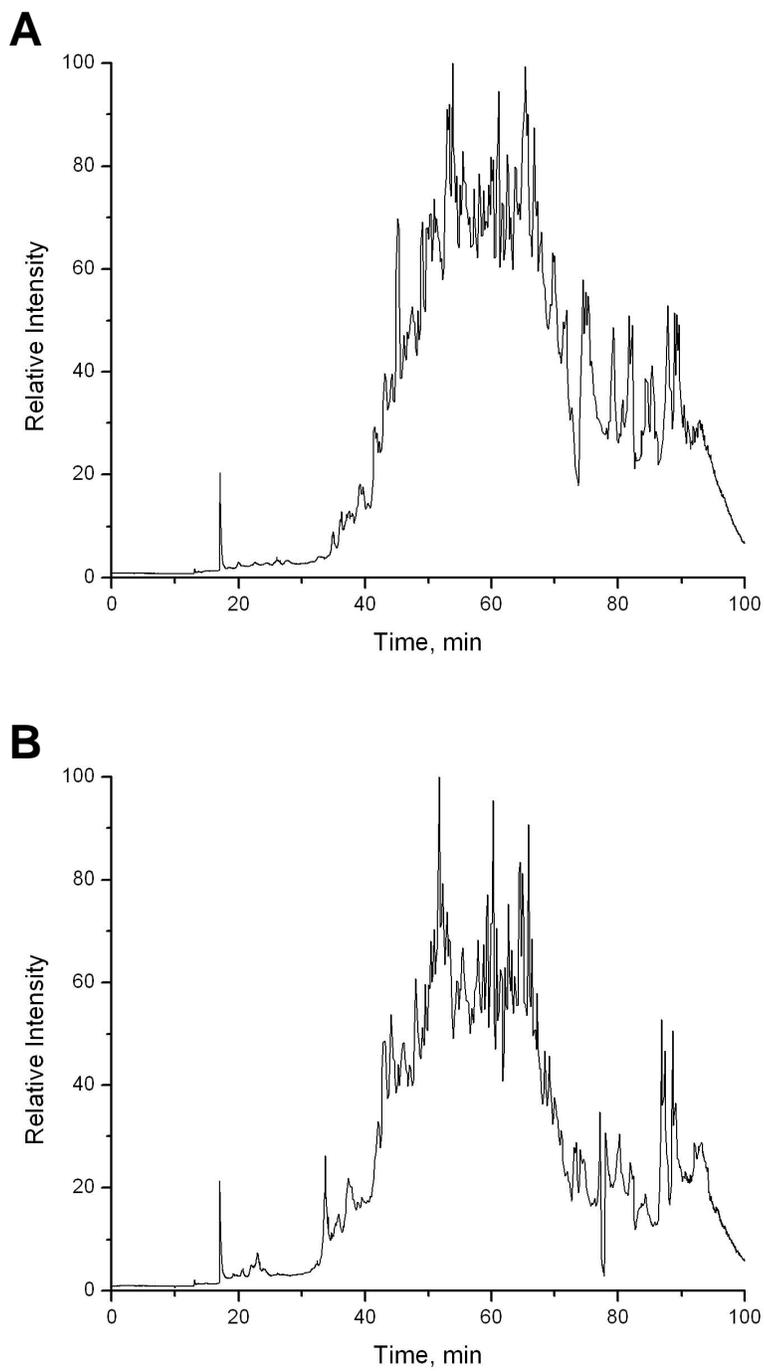
Figure 20: Total ion current (TIC) of the LC-MS experiment using A. peptides extracted from band 17 of the MXR MCF-7 SDS-PSGE gel and B. peptides extracted from band 19 of the MXR MCF-7 SDS-PSGE gel.

The chromatography performed using the LC-Packing system has excellent resolving capability with peak widths of 1 min or less. This provides good signal intensity, however the tandem mass spectrometry data must be collected as fast as possible to obtain multiple peptide mass measurements. The computer performs a 1 second survey scan to identify the m/z and charge states of the peptides to be analyzed, followed by 3 MS/MS scans of 3 seconds/scan on the 3 most intense doubly or triply charged peptides present. Therefore 1 cycle of this process spans 10 seconds. Once a MS/MS mass measurement is made on a peptide of a particular m/z value, that particular mass is ignored for 1 minute to make sure that the same 3 most intense peaks do not get picked repeatedly while some of the less intense peaks get ignored. This process is commonly called dynamic exclusion.

Figure 21 shows 1 min survey scans from bands 17 and 19. In each of these cases the peaks are well resolved with little overlap due to the chromatographic separation upstream from the mass spectrometry analysis. The doubly and triply charged peptides are selected from these survey scans for 3 second MS/MS scans with minimal repetition as explained above. The insets of **Figure 21 A** and **B** show the expanded views of these survey scans focusing on the 585.8 and 644.8 doubly charged peptides.

Due to the limited time window in which peptides eluting from columns can be analyzed by the mass spectrometer in LC-MS experiments, computer automation has become a major factor in the data collection during these types of experiments.
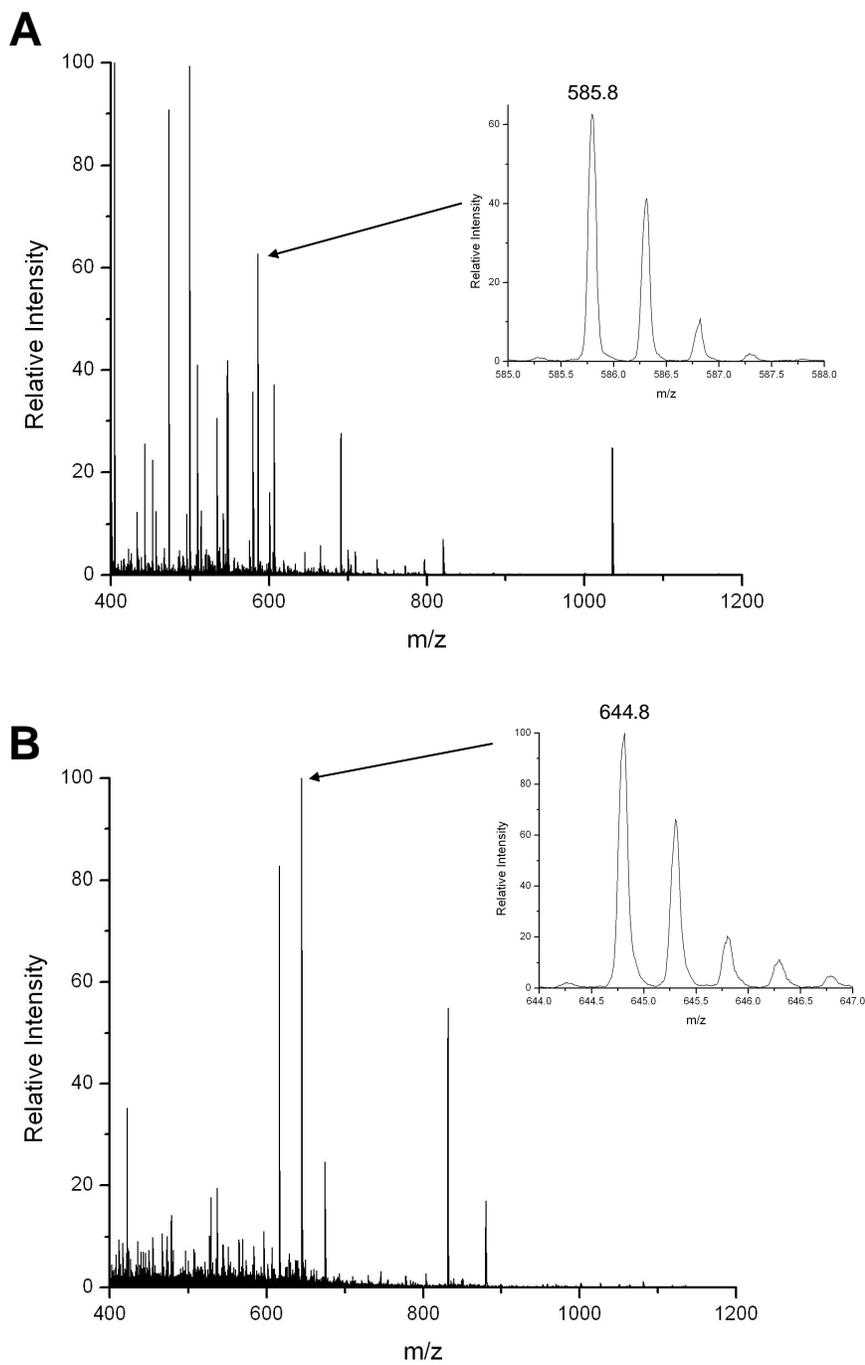
Figure 21: A. Survey scan of the band 17 LC-MS experiment from 53 to 54 min with expanded view if the doubly charged 644.8 peptide in the inset spectrum. B. Survey scan of the band 19 LCMS experiment from 47.5 to 48.5 min with expanded view if the doubly charged 644.8 peptide in the inset spectrum.

The software used can quickly differentiate between the doubly and triply charged peptides, which generate MS/MS spectrum useful for protein identification, and the peptides of higher charges, which generate less information rich spectrum and are harder to analyze. These programs can also exclude peaks corresponding to non-peptide species. This ability to differentiate between these types of molecular ions allows the mass spectrometer to collect data more efficiently and therefore increase the chances for protein identifications.

Computer software is not only critical for the acquisition of data during LC-MS experiments but computer algorithms are also indispensable for the analysis of the data acquired. Liquid chromatography mass spectrometry experiments can generate enormous amounts of data, and especially in the case of a shotgun analysis where proteins are digested before any protein separation is performed, many peptides from the same protein can be separated across many different fractions. Commercial programs can analyze the LC-MS data and recompile it into a list of proteins identified from several different experiments.

These automated analyses use sophisticated computer algorithms to obtain protein identifications by comparing the experimental MS/MS fragmentation data with the theoretical MS/MS fragmentation data of peptides generated from proteins in a protein database. This MS/MS ion search combined with any additional information, such as the parent peptide molecular weight, the particular enzyme used for the

digestion, and species information, if available, is then used to identify which protein the peptide originated from.

Another factor which helps in the automated identification process is the identification of immonium ions in the spectrum. Immonium ions are formed by the multiple cleavages within the peptide. Each contains an amino group and a side chain from an amino acid residue in the peptide. While this information does not help in identifying the primary sequence of the peptide, each immonium ion reveals the presence of its parent amino acid and consequently aids in the peptide identifications by corroborating the presence of the amino acid in the sequence of the peptide.

About 10,000 MS/MS scans were performed on peptides automatically selected in the LC-MS experiments on the peptides recovered from the in-gel digestion on the 28 gel bands. **Figures 22** and **23** show the results of the automated identification from the 585.8 and 644.8 doubly charged peptides shown in **Figures 21 A** and **B** from bands 17 and 19 respectively.

The upper panes of **Figures 22** and **23** show the actual MS/MS fragmentation data while the lower panes show theoretical fragmentation data from the peptide tentatively identified using the seven ions that occur most frequently when activated by low energy multiple collisions. The numbered values are the expected ions present based on the identification assigned by the program and the numbers

+TOF Product (585.8): Experiment 2, 54.212 min from 6-25-04Mitox 40ug Band 17.wiff   Max. 1488.0 counts.
a=3.56891980677738420e-004, t0=3.71835173391382060e+001

Intensity, counts — m/z, amu

645.2434
758.3764
871.4963
300.1596
86.0772
227.1623
413.2162
558.3079
199.1648
385.2303
526.3385
481.3093

Controls

| Reset to Default... | Set as Default | | Sequence | Tag |

Sequences and Tags   +TOF Product (585.8): Experiment 2, 54.212 min from 6-25-04Mitox 40ug Band 17.wiff

Fragments   [Precursor MW = 1169.5696, Charge = 2]   41 of 83 matched - 49%

H- ADLLLSTQPGR(-0.0708)   -OH 0.0000   Ion charge: 1

	Residue	Mass	Immonium	a	a-NH3	b	b-NH3	y	y-NH3
1	A, Ala	71.0371	44.0495	44.0495	27.0229	72.0444	55.0178	1170.5769	1153.5503
2	D, Asp	115.0269	88.0393	159.0764	142.0499	187.0713	170.0448	1099.5398	1082.5132
3	L, Leu	113.0841	86.0964	272.1605	255.1339	300.1554	283.1288	984.5128	967.4863
4	L, Leu	113.0841	86.0964	385.2445	368.2180	413.2395	396.2129	871.4288	854.4022
5	L, Leu	113.0841	86.0964	498.3286	481.3021	526.3235	509.2970	758.3447	741.3181
6	S, Ser	87.0320	60.0444	585.3606	568.3341	613.3556	596.3290	645.2606	628.2341
7	T, Thr	101.0477	74.0600	686.4083	669.3818	714.4032	697.3767	558.2286	541.2021
8	Q, Gln	128.0586	101.0709	814.4669	797.4403	842.4618	825.4353	457.1809	440.1544
9	P, Pro	97.0528	70.0651	911.5197	894.4931	939.5146	922.4880	329.1223	312.0958
10	G, Gly	57.0215	30.0338	968.5411	951.5146	996.5360	979.5095	232.0696	215.0430
11	R, Arg	156.1011	129.1135	1124.6422	1107.6157	1152.6371	1135.6106	175.0481	158.0216
12	(-0.0708)	-0.0708	n/a	1124.5714	1107.5448	1152.5663	1135.5398	18.9470	1.9205

Figure 22: Automated sequence identification of the doubly charged peptide 585.8 from band 17 using the integrated Analyst QS software with ProID. Fragment ions matching the theoretical fragmentation data are highlighted in yellow.
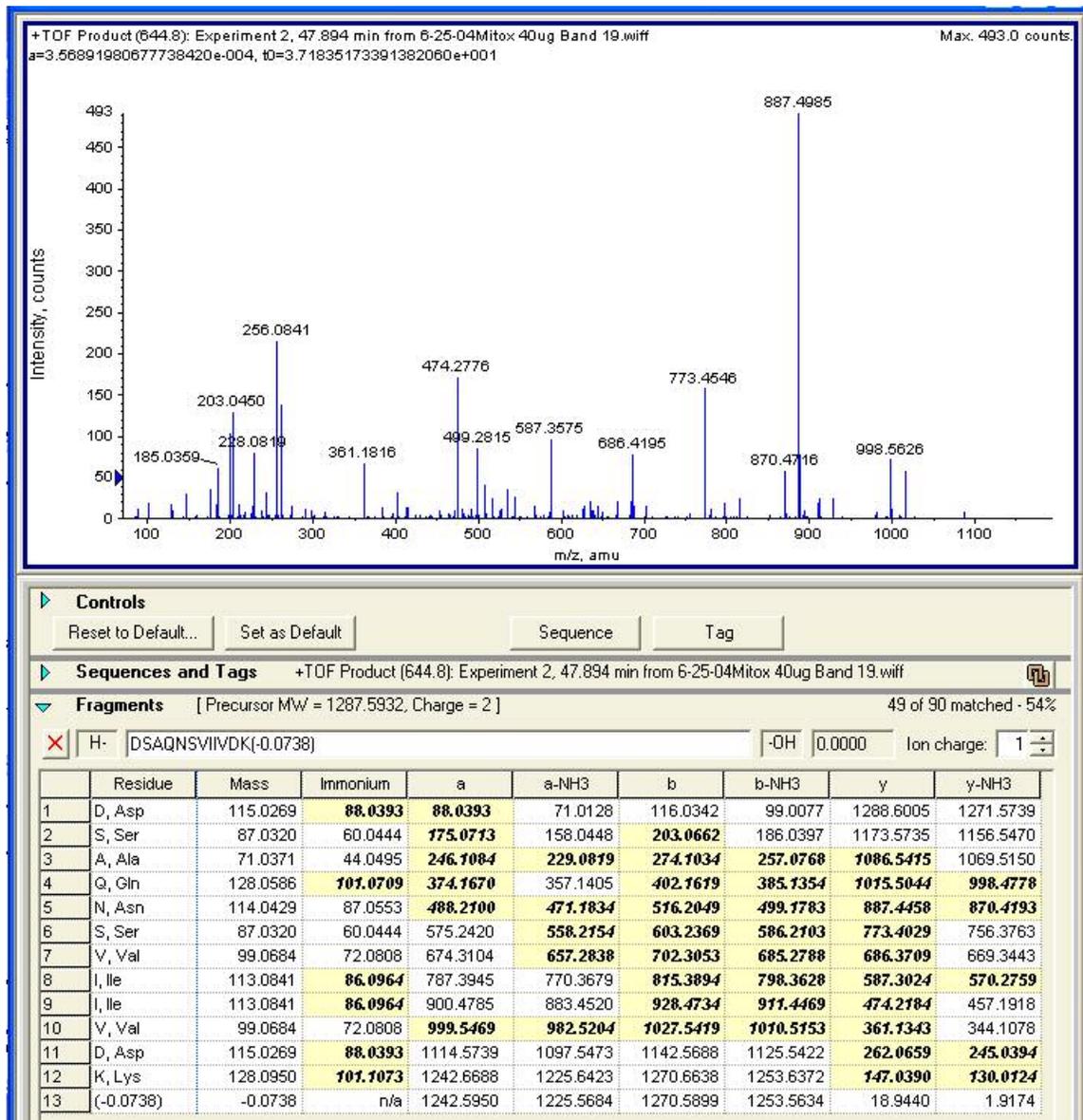
Figure 23: Automated sequence identification of the doubly charged peptide 644.8 from band 19 using the integrated Analyst QS software with ProID. Fragment ions matching the theoretical fragmentation data are highlighted in yellow.

highlighted in yellow denote experimental values which correspond to theoretical values. The predominant species present in the fragmentation data of most of these experiments are the b- and y-ions. The resulting peptide identities are assigned probability scores in the form of confidence percentages.

The practical application of this technology still requires human interpretation of the resulting automated data analysis. This automated analysis of LC-MS data can propose multiple protein identifications and a set of parameter restrictions must be set to filter out the lower probability hits. All low scoring peptide sequences and proteins identified from only 1 peptide identification were excluded from the list of proteins identified, no mater what the peptide confidence score. By including only peptide identifications of high confidence and only counting protein identifications based on 2 or more peptides we can greatly reduce the number of false positives.

In addition, each protein identification was manually checked. Peptide sequence tags were used to manually validate the identity of each of the proteins identified. An example of this procedure is shown in **Figure 24 A and B**. The same peptides analyzed using the m/z 585.8 and 644.8 doubly charged ions from bands 17 and 19 were sequenced manually as is shown in the Figure. The amino acid sequence tags "SLLL" and "VSNQ" were identified from the y-ion sequence information and this data, along with the name of the enzyme used in the protein cleavage and the parent peptide mass, were used to identify the peptides, and subsequently the proteins they came from. The m/z 585.8 peptide was found to be from the 4F2 cell-surface
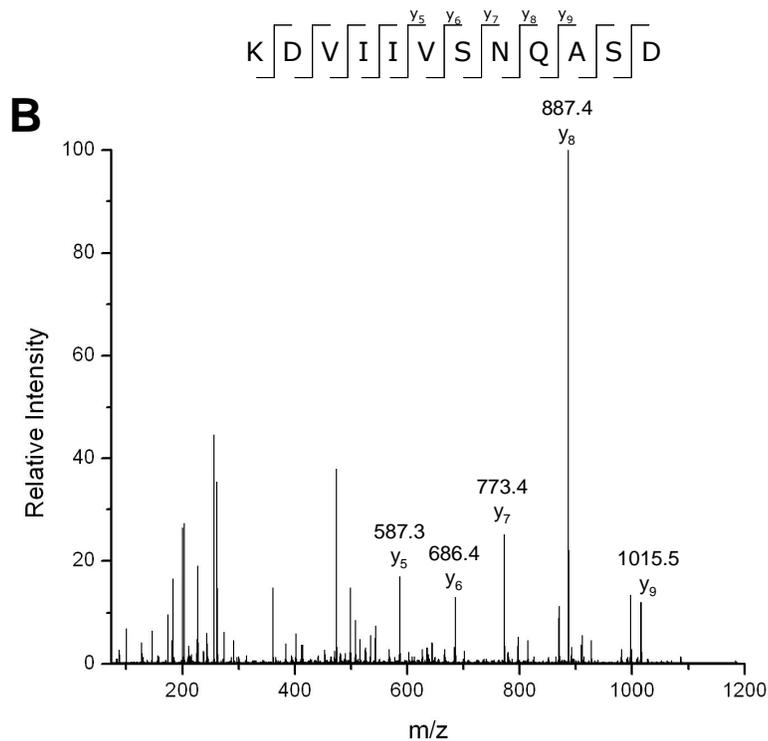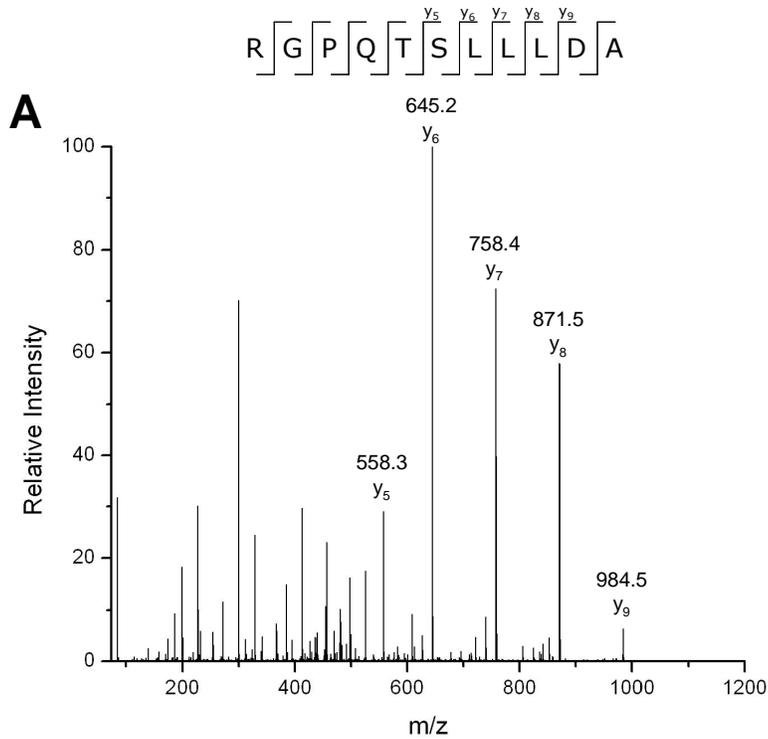
Figure 24: A. Amino acid sequence tag "SLLL" obtained manually by sequencing the y5 to y9 fragment ions from the 585.8 peptide from band 17. B. Amino acid sequence tag "VSNQ" obtained manually by sequencing the y5 to y9 fragment ions from the 644.8 peptide from band 19.

antigen (sp|P08195), and the m/z 644.8 peptide was found to be from the

Transferrin receptor protein 1 (sp|P02786).

One of the strengths of the software is the ability of the program to recompile the

protein information from many experiments into a summary of all the proteins

present from many different analyses done over a period of time. Using this

function we can come up with a protein list of all the proteins identified from all 28

of our gel bands. Once all of the proteins are manually validated we produce a final

list of proteins identified. **Table 5** shows the list of all proteins identified from the

basolateral plasma membrane fraction isolated from the MCF-7 cell line along with

their subcellular location and the number of peptides used to make the identification.

A total of 540 distinct proteins was identified from 3227 unique peptide

identifications. **Figure 25** shows a graphical representation of the subcellular

locations of each of the proteins as defined by the SwissProt annotation and Gene

Ontology assignments, along with the number of the 3227 sequenced peptides that

originate from proteins from the plasma membrane. **Figure 26** shows the 100 proteins

most reliably identified, based on their scores and the number of peptides used to

make each identification. The 1467 peptides used to identify these 100 proteins

account for almost half of the peptides sequenced.

Accession #	Protein Name	Location	# of Peptides
sp|Q00610	Clathrin heavy chain 1	PM	54
sp|P06756	Integrin alpha-V precursor	PM	40
sp|P05023	Sodium/potassium-transporting ATPase alpha-1 chain	PM	38
sp|P08195	4F2 cell-surface antigen heavy chain	PM	33
tr|O75054	Hypothetical protein KIAA0466	Hypo Memb	32
sp|P02786	Transferrin receptor protein 1	PM	31
sp|P20020	Plasma membrane calcium-transporting ATPase 1	PM	31
sp|P17301	Integrin alpha-2 precursor	PM	29
sp|P05787	Keratin, type II cytoskeletal 8	Cytoskeleton	27
sp|P18084	Integrin beta-5 precursor	PM	27
tr|Q9UIU0	Dihydropyridine receptor alpha 2 subunit	PM	25
sp|P13639	Elongation factor 2 (EF-2)	Cytoplasm	23
tr|Q8IX16	ATP-binding cassette protein ABCG2	PM	23
sp|P54760	Ephrin type-B receptor 4 precursor	PM	21
sp|P10586	LAR protein precursor	PM	20
sp|Q9P2B2	Prostaglandin F2 receptor negative regulator precursor	PM	20
sp|P05218	Tubulin beta-5 chain	Cytoskeleton	19
sp|Q9NZM1	Myoferlin	PM	19
sp|O95782	Adapter-related protein complex 2 alpha 1 subunit	PM	18
sp|P05141	ADP,ATP carrier protein, fibroblast isoform	PM	18
sp|Q14254	Flotillin-2	PM	18
tr|Q7Z3V1	Hypothetical protein DKFZp686D0452	Hypo Memb	17
sp|P01031	Complement C5 precursor	Secreted	16
sp|P05217	Tubulin beta-2 chain	Cytoskeleton	16
sp|P08069	Insulin-like growth factor I receptor precursor	PM	16
sp|P12236	ADP,ATP carrier protein, liver isoform T2	Mitochondria	16
sp|P13645	Keratin, type I cytoskeletal 10	Cytoskeleton	16
sp|P55011	Solute carrier family 12 member 2	PM	16
sp|O15394	Neural cell adhesion molecule 2 precursor	PM	15
sp|P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit	ER	15
sp|P05783	Keratin, type I cytoskeletal 18	Cytoskeleton	15
sp|P14618	Pyruvate kinase, M1 isozyme	Cytoplasm	15
sp|P21851	Adapter-related protein complex 2 beta 1 subunit	PM	15
sp|P04626	Receptor protein-tyrosine kinase erbB-2 precursor	PM	14
sp|P04895	Guanine nucleotide-binding protein	PM	14

Accession #	Protein Name	Location	# of Peptides
	G(S), alpha subunit		
sp|P04899	Guanine nucleotide-binding protein G(i), alpha-2 subunit	PM	14
sp|P50993	Sodium/potassium-transporting ATPase alpha-2 chain	PM	14
sp|Q16720	Plasma membrane calcium-transporting ATPase 3	PM	14
tr|Q14433	Guanine nucleotide-binding protein G-s-alpha-3	PM	14
tr|Q8TBC0	Similar to GNAS complex locus	PM	14
sp|O00159	Myosin Ic	Cytoplasm	13
sp|P04264	Keratin, type II cytoskeletal 1	Cytoskeleton	13
sp|P07900	Heat shock protein HSP 90-alpha	Cytoplasm	13
sp|P27105	Erythrocyte band 7 integral membrane protein	PM	13
sp|P35908	Keratin, type II cytoskeletal 2 epidermal	Cytoskeleton	13
tr|Q8IWA5	CTL2 protein	PM	13
tr|Q9UIW2	NOV/plexin-A1 protein	PM	13
sp|O94973	Adapter-related protein complex 2 alpha 2 subunit	PM	12
sp|P01024	Complement C3 precursor	Secreted	12
sp|P05209	Tubulin alpha-1 chain	Cytoskeleton	12
sp|P12235	ADP,ATP carrier protein, heart	PM	12
sp|P24539	ATP synthase B chain, mitochondrial precursor	Mitochondria	12
sp|P27824	Calnexin precursor	ER	12
sp|P51148	Ras-related protein Rab-5C	PM	12
sp|Q13740	CD166 antigen precursor	PM	12
sp|Q9BQE3	Tubulin alpha-6 chain	Cytoskeleton	12
sp|Q9UL26	Ras-related protein Rab-22A	PM	12
sp|Q92508	Hypothetical protein KIAA0233	Hypo Memb	12
tr|Q8WWI5	Choline transporter-like protein 1, splice variant a	PM	12
sp|P02304	Histone H4	Nuclei	11
sp|P06576	ATP synthase beta chain, mitochondrial precursor	Mitochondria	11
sp|P08238	Heat shock protein HSP 90-beta	Cytoplasm	11
sp|P08727	Keratin, type I cytoskeletal 19	Cytoskeleton	11
sp|P20172	Clathrin coat assembly protein AP50	PM	11
sp|P51149	Ras-related protein Rab-7	PM	11
tr|Q9ULH0	Hypothetical protein KIAA1250	Hypo Memb	11
sp|O88386	Ras-related protein Rab-10	PM	10
sp|P02570	Actin, cytoplasmic 1	Cytoskeleton	10
sp|P21796	Voltage-dependent anion-selective channel protein 1	PM	10
sp|P35287	Ras-related protein Rab-14	PM	10
sp|Q9H0U4	Ras-related protein Rab-1B	PM	10
tr|Q16702	Fatty acid synthase	Cytoplasm	10
tr|Q8TD43	Cation channel TRPM4B	PM	10

Accession #	Protein Name	Location	# of Peptides
tr|Q99623	B-cell receptor associated protein	Cytoplasm	10
sp|O00161	Synaptosomal-associated protein 23	PM	9
sp|P01111	Transforming protein N-Ras	PM	9
sp|P01112	Transforming protein p21	PM	9
sp|P04844	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit	ER	9
sp|P05026	Sodium/potassium-transporting ATPase beta-1 chain	PM	9
sp|P05215	Tubulin alpha-4 chain	Cytoskeleton	9
sp|P06733	Alpha enolase	Cytoplasm	9
sp|P08754	Guanine nucleotide-binding protein G(k), alpha subunit (G(i) alpha-3)	PM	9
sp|P20339	Ras-related protein Rab-5A	PM	9
sp|P25705	ATP synthase alpha chain, mitochondrial precursor	Mitochondria	9
sp|P35527	Keratin, type I cytoskeletal 9	Cytoskeleton	9
sp|Q15758	Neutral amino acid transporter B(0) (ATB(0))	PM	9
sp|Q8TAA9	Vang-like protein 1	PM	9
sp|Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar2	PM	9
sp|P17082	Ras-related protein R-Ras2	PM	9
sp|P11717	Cation-independent mannose-6-phosphate receptor precursor	PM	8
sp|O75955	Flotillin-1	PM	8
sp|P04406	Glyceraldehyde 3-phosphate dehydrogenase, liver	Cytoplasm	8
sp|P08886	Ras-related protein Rab-2A	PM	8
sp|P11142	Heat shock cognate 71 kDa protein	Cytoplasm	8
sp|P15154	Ras-related C3 botulinum toxin substrate 1	PM	8
sp|P16422	Tumor-associated calcium signal transducer 1 precursor	PM	8
sp|P16587	ADP-ribosylation factor 3	PM	8
sp|P22392	Nucleoside diphosphate kinase B	Nuclei	8
sp|P24410	Ras-related protein Rab-11A	PM	8
sp|P26438	ADP-ribosylation factor 6	PM	8
sp|P29316	60S ribosomal protein L23a	Ribosome	8
sp|P35232	Prohibitin	Cytoplasm	8
sp|P48669	Keratin, type II cytoskeletal 6F	Cytoskeleton	8
sp|P54709	Sodium/potassium-transporting ATPase beta-3 chain	PM	8
sp|Q00325	Phosphate carrier protein, mitochondrial precursor	PM	8
sp|Q02978	Mitochondrial 2-oxoglutarate	PM	8
sp|Q8WTV0	Scavenger receptor class B member 1	PM	8
sp|Q9NP72	Ras-related protein Rab-18	PM	8
sp|Q9UIQ6	Leucyl-cystinyl aminopeptidase	PM	8
tr|Q14160	Hypothetical protein KIAA0147	Hypothetical	8

Accession #	Protein Name	Location	# of Peptides
tr|Q86X29	Similar to liver-specific bHLH-Zip transcription factor	Hypo Memb	8
tr|Q8NE01	Cyclin M3	Membrane	8
tr|Q96AG4	Hypothetical protein (Similar to expressed sequence AA959742)	Hypo Memb	8
tr|Q9HCI0	Hypothetical protein KIAA1592	Hypo Memb	8
sp|Q9Y5L3	Ectonucleoside triphosphate diphosphohydrolase 2	PM	8
sp|P07947	Proto-oncogene tyrosine-protein kinase YES	PM	8
sp|Q14204	Dynein heavy chain, cytosolic	Cytoplasm	8
sp|P21333	Filamin A	Cytoskeleton	7
sp|O14672	ADAM 10 precursor	PM	7
sp|P02278	Histone H2B.a	Nuclei	7
sp|P02568	Actin, alpha skeletal muscle	Cytoskeleton	7
sp|P04765	Eukaryotic initiation factor 4A-I	Cytoplasm	7
sp|P04920	Anion exchange protein 2	PM	7
sp|P06749	Transforming protein RhoA	PM	7
sp|P06899	Histone H2B.r	Nuclei	7
sp|P08107	Heat shock 70 kDa protein 1	Cytoplasm	7
sp|P08133	Annexin A6	Cytoplasm	7
sp|P09525	Annexin A4	Cytoplasm	7
sp|P17964	Ras-related protein Rap-2b	PM	7
sp|P18085	ADP-ribosylation factor 4	PM	7
sp|P20340	Ras-related protein Rab-6A	PM	7
sp|P20648	Potassium-transporting ATPase alpha chain 1	PM	7
sp|P23396	40S ribosomal protein S3	Ribosome	7
sp|P29312	14-3-3 protein zeta	Cytoplasm	7
sp|P33527	Multidrug resistance-associated protein 1	PM	**7**
sp|P35239	Ras-related protein Rab-5B	PM	7
sp|P42167	Thymopoietin, isoforms beta	Nuclei	7
sp|P50895	Lutheran blood group glycoprotein precursor	PM	7
sp|P54753	Ephrin type-B receptor 3 precursor	PM	7
sp|P56159	GDNF family receptor alpha 1 precursor	PM	7
sp|Q01650	Large neutral amino acids transporter small subunit 1	PM	7
sp|Q13308	Tyrosine-protein kinase-like 7 precursor	PM	7
sp|Q14126	Desmoglein 2 precursor	PM	7
sp|Q15907	Ras-related protein Rab-11B	PM	7
sp|Q9H9B4	Sideroflexin 1	Mitochondria	7
sp|Q9Y277	Voltage-dependent anion-selective channel protein 3	Mitochondria	7
sp|Q9Y5M8	Signal recognition particle receptor beta subunit	ER	7
tr|O00648	Protein tyrosine phosphatase	Hypothetical	7

Accession #	Protein Name	Location	# of Peptides
	PTPCAAX1		
tr|O94980	Hypothetical protein KIAA0906	Hypo Memb	7
tr|Q07065	P63 protein	Membrane	7
tr|Q7Z7Q4	Tumor-associated calcium signal transducer 2	PM	7
tr|Q8NFA6	Transmembrane receptor PTK7-4	PM	7
tr|Q96QB2	Sodium-independent neutral amino acid transporter LAT1	PM	7
tr|Q9Y6C9	HSPC032 (Mitochondrial carrier homolog 2)	Membrane	7
sp|P02649	Apolipoprotein E precursor	Secreted	7
sp|P10643	Complement component C7 precursor	Secreted	7
sp|P10113	Ras-related protein Rap-1A	PM	7
sp|Q06830	Peroxiredoxin 1	Cytoplasm	7
sp|P07358	Complement component C8 beta chain precursor	Secreted	7
sp|O43570	Carbonic anhydrase XII precursor	PM	6
sp|O60716	Catenin delta-1	Cytoplasm	6
sp|O75947	ATP synthase D chain, mitochondrial	Mitochondria	6
sp|O95858	Tetraspan NET-7	PM	6
sp|P00403	Cytochrome c oxidase polypeptide II	Mitochondria	6
sp|P04075	Fructose-bisphosphate aldolase A	Cytoplasm	6
sp|P06744	Glucose-6-phosphate isomerase	Cytoplasm	6
sp|P07355	Annexin A2	PM	6
sp|P11016	Guanine nucleotide-binding protein G(I)	PM	6
sp|P11476	Ras-related protein Rab-1A	PM	6
sp|P12259	Coagulation factor V precursor	Secreted	6
sp|P23528	Cofilin, non-muscle isoform	Cytoskeleton	6
sp|P39656	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	ER	6
sp|P45880	Voltage-dependent anion-selective channel protein 2	Mitochondria	6
sp|P49411	Elongation factor Tu, mitochondrial precursor	Mitochondria	6
sp|P49755	Transmembrane protein Tmp21 precursor	PM	6
sp|P50395	Rab GDP dissociation inhibitor beta	PM	6
sp|Q07020	60S ribosomal protein L18	Ribosome	6
sp|Q12907	Vesicular integral-membrane protein VIP36 precursor	ER	6
sp|Q14344	Guanine nucleotide-binding protein, alpha-13 subunit	PM	6
sp|Q92542	Nicastrin precursor	PM	6
sp|Q9UL25	Ras-related protein Rab-21	PM	6
sp|Q9Y4D1	Disheveled associated activator of morphogenesis 1	Cytoplasm	6
tr|Q8NBN5	Thioredoxin domain containing protein 1	PM	6

Accession #	Protein Name	Location	# of Peptides
tr|Q9BTT5	Similar to NADH dehydrogenase	Mitochondria	6
tr|Q9Y639	Stromal cell-derived receptor-1 alpha	PM	6
sp|P54707	Potassium-transporting ATPase alpha chain 2	PM	6
sp|Q08211	ATP-dependent RNA helicase A	Nuclei	6
sp|P08758	Annexin A5	PM	6
sp|O43278	Kunitz-type protease inhibitor 1 precursor	Secreted	6
sp|P09526	Ras-related protein Rap-1b	PM	6
sp|P23284	Peptidyl-prolyl cis-trans isomerase B precursor	ER	6
sp|Q9Y4W6	AFG3-like protein 2	Mitochondria	6
sp|P26006	Integrin alpha-3 precursor	PM	6
sp|P07339	Cathepsin D precursor	Cytoplasm	6
tr|Q7Z341	Hypothetical protein DKFZp686J08113	Hypo Memb	5
sp|O14662	Syntaxin 16 (Syn16)	Golgi	5
sp|O95292	Vesicle-associated membrane protein-associated protein	PM	5
sp|O95716	Ras-related protein Rab-3D	PM	5
sp|P04901	Guanine nucleotide-binding protein G(I)	PM	5
sp|P07996	Thrombospondin 1 precursor	PM	5
sp|P10301	Ras-related protein R-Ras	PM	5
sp|P10809	60 kDa heat shock protein, mitochondrial precursor	Mitochondria	5
sp|P15880	40S ribosomal protein S2	Ribosome	5
sp|P18124	60S ribosomal protein L7	Ribosome	5
sp|P21926	CD9 antigen	PM	5
sp|P24407	Ras-related protein Rab-8	PM	5
sp|P25388	Guanine nucleotide-binding protein beta subunit-like protein 12.3	PM	5
sp|P26641	Elongation factor 1-gamma	Cytoplasm	5
sp|P29992	Guanine nucleotide-binding protein G(Y), alpha subunit	PM	5
sp|P35221	Alpha-1 catenin	Cytoskeleton	5
sp|P35238	Rho-related GTP-binding protein RhoG	PM	5
sp|P35613	Basigin precursor	PM	5
sp|P50148	Guanine nucleotide-binding protein G(q), alpha subunit	PM	5
sp|P50443	Sulfate transporter	PM	5
sp|P51153	Ras-related protein Rab-13	PM	5
sp|P51571	Translocon-associated protein, delta subunit precursor	ER	5
sp|P51805	Plexin A3 precursor	PM	5
sp|P60033	CD81 antigen	PM	5
sp|Q99808	Equilibrative nucleoside transporter 1	PM	5
sp|Q9UBM7	7-dehydrocholesterol reductase	ER	5
sp|Q9Y512	SAM50-like protein CGI-51	Mitochondria	5

Accession #	Protein Name	Location	# of Peptides
sp|Q9Y5Y6	Suppressor of tumorigenicity 14	PM	5
sp|Q9Y624	Junctional adhesion molecule 1 precursor	PM	5
sp|Q9Y666	Solute carrier family 12 member 7	PM	5
tr|O75396	Vesicle trafficking protein SEC22B	ER	5
tr|O75870	PTPsigma	Cytoplasm	5
tr|Q7Z5X0	Semaphorin 4C	PM	5
tr|Q8IXI1	Rho 2	Mitochondria	5
tr|Q8N271	Hypothetical protein FLJ33856	Hypo Memb	5
tr|Q8NC51	Hypothetical protein FLJ90489	Hypothetical	5
tr|Q8NCB6	Hypothetical protein FLJ90368	Hypo Memb	5
tr|Q8NI87	Phosphoglycerete kinase 1	Cytoplasm	5
tr|Q9BTZ7	Hypothetical protein	Hypothetical	5
tr|Q9NX63	Hypothetical protein FLJ20420	Hypothetical	5
tr|Q9UEI6	Polio virus related protein 2, alpha isoform	PM	5
sp|O15440	Multidrug resistance-associated protein 5	PM	5
sp|P09058	40S ribosomal protein S8	Ribosome	5
sp|P11413	Glucose-6-phosphate 1-dehydrogenase	Cytoplasm	5
sp|P26373	60S ribosomal protein L13 (Breast basic conserved protein 1)	Ribosome	5
sp|P27348	14-3-3 protein tau	Cytoplasm	5
sp|P46781	40S ribosomal protein S9	Ribosome	5
sp|Q08722	Leukocyte surface antigen CD47 precursor	PM	5
sp|Q8WXE9	Stonin 2	PM	5
tr|Q8TE01	DERP12	Cytoplasm	5
tr|Q9BSY2	C9orf89 protein	Hypo Memb	5
tr|Q9NVJ2	Hypothetical protein FLJ10702	Hypo Memb	5
sp|P07360	Complement component C8 gamma chain precursor	Secreted	5
sp|P12931	Proto-oncogene tyrosine-protein kinase Src	PM	5
sp|P42655	14-3-3 protein epsilon	Cytoplasm	5
sp|Q86UP2	Kinectin	ER	5
sp|Q9NRW1	Ras-related protein Rab-6B	PM	5
tr|Q7YCE6	Cytochromec oxidase subunit II	Mitochondria	5
sp|P00354	Glyceraldehyde 3-phosphate dehydrogenase, muscle	Cytoplasm	5
sp|P04720	Elongation factor 1-alpha 1	Cytoplasm	5
sp|P21378	Microsomal signal peptidase 18 kDa subunit	PM	5
sp|P29317	Ephrin type-A receptor 2 precursor	PM	5
sp|Q05639	Elongation factor 1-alpha 2	Nuclei	5
tr|Q9BTS0	Hypothetical protein	Hypo Memb	5
sp|P00338	L-lactate dehydrogenase A chain	Cytoplasm	5
sp|P51531	Possible global transcription activator SNF2L2	Nuclei	5

Accession #	Protein Name	Location	# of Peptides
sp|O00264	Membrane associated progesterone receptor component 1	PM	4
sp|O15439	Multidrug resistance-associated protein 4	PM	**4**
sp|O15551	Claudin-3	PM	4
sp|O95471	Claudin-7	PM	4
sp|P01121	Transforming protein RhoB	PM	4
sp|P02261	Histone H2A.c	Nuclei	4
sp|P02748	Complement component C9 precursor	PM	4
sp|P02768	Serum albumin precursor	Secreted	4
sp|P04792	Heat shock 27 kDa protein	Cytoplasm	4
sp|P11021	78 kDa glucose-regulated protein precursor	ER	4
sp|P11166	Solute carrier family 2, facilitated glucose transporter, member 1	PM	4
sp|P13671	Complement component C6 precursor	Secreted	4
sp|P15559	NAD(P)H dehydrogenase 1	Cytoplasm	4
sp|P20337	Ras-related protein Rab-3B	PM	4
sp|P20700	Lamin B1	Nuclei	4
sp|P22087	Fibrillarin	Nuclei	4
sp|P22102	Trifunctional purine biosynthetic protein adenosine-3	Cytoplasm	4
sp|P30050	60S ribosomal protein L12	Ribosome	4
sp|P31930	Ubiquinol-cytochrome C reductase complex core protein I	Mitochondria	4
sp|P34897	Serine hydroxymethyltransferase, mitochondrial precursor	Mitochondria	4
sp|P48735	Isocitrate dehydrogenase [NADP], mitochondrial precursor	Mitochondria	4
sp|P50991	T-complex protein 1, delta subunit	Cytoplasm	4
sp|P57088	DB83 protein	Hypo Memb	4
sp|Q04721	Neurogenic locus notch homolog protein 2 precursor	PM	4
sp|Q13724	Mannosyl-oligosaccharide glucosidase	ER	4
sp|Q14118	Dystroglycan precursor	PM	4
sp|Q14165	Hypothetical protein KIAA0152	Hypo Memb	4
sp|Q15005	Microsomal signal peptidase 25 kDa subunit	PM	4
sp|Q15836	Vesicle-associated membrane protein 3	PM	4
sp|Q16625	Occludin - Homo sapiens (Human).	PM	4
sp|Q8NB49	Potential phospholipid-transporting ATPase IG	PM	4
sp|Q9HDC9	Adipocyte plasma membrane-associated protein	PM	4
tr|O15498	SNARE protein Ykt6	PM	4
tr|O60376	P1.11659_4	Hypo Memb	4
tr|Q14980	Nuclear mitotic apparatus protein 1	Nuclei	4
tr|Q7Z3X1	Hypothetical protein DKFZp686A18156	Hypo Memb	4

Accession #	Protein Name	Location	# of Peptides
tr|Q7Z7A6	PPR motif-containing protein	Mitochondria	4
tr|Q8IUI5	Similar to butyrate-induced transcript 1	Hypo Memb	4
tr|Q96E39	Similar to RNA binding motif protein, X chromosome	Nuclei	4
tr|Q96FY2	Stomatin-like 2	PM	4
tr|Q9H8M5	Hypothetical protein FLJ13417	Hypo Memb	4
sp|O75695	XRP2 protein	Cytoplasm	4
sp|P01118	Transforming protein p21b	PM	4
sp|P06213	Insulin receptor precursor	PM	4
sp|P35214	14-3-3 protein gamma	Cytoplasm	4
sp|Q12846	Syntaxin 4	PM	4
sp|Q15286	Ras-related protein Rab-35	PM	4
sp|Q92896	Golgi apparatus protein 1 precursor	PM	4
sp|Q9NS69	Mitochondrial import receptor subunit TOM22 homolog	Mitochondria	4
sp|O43760	Synaptogyrin 2	PM	4
sp|P04643	40S ribosomal protein S11	Ribosome	4
sp|P05092	Peptidyl-prolyl cis-trans isomerase A	Cytoplasm	4
sp|P18621	60S ribosomal protein L17	Ribosome	4
sp|P25232	40S ribosomal protein S18	Ribosome	4
sp|P31946	14-3-3 protein beta	Cytoplasm	4
sp|Q04917	14-3-3 protein eta	Cytoplasm	4
sp|Q14964	Ras-related protein Rab-39A	PM	4
sp|Q9NX76	Chemokine-like factor super family member 6	PM	4
tr|O75592	Protein associated with Myc	Cytoplasm	4
tr|Q9P134	PRO3078	Cytoplasm	4
sp|P13646	Keratin, type I cytoskeletal 13	Cytoskeleton	4
sp|P29401	Transketolase	Cytoplasm	4
sp|P78371	T-complex protein 1, beta subunit	Cytoplasm	4
sp|Q14152	Eukaryotic translation initiation factor 3 subunit 10	Cytoplasm	4
sp|Q92982	Ninjurin 1	PM	4
sp|P12750	40S ribosomal protein S4, X isoform	Ribosome	4
sp|P51587	Breast cancer type 2 susceptibility protein	Nuclei	4
tr|Q8IWE4	Hypothetical protein MGC48972	Hypothetical	4
tr|Q8NBN2	Hypothetical protein NT2RP2002695	Hypo Memb	4
sp|O75369	Filamin B	Cytoskeleton	3
sp|P04114	Apolipoprotein B-100 precursor	Secreted	3
tr|Q7Z5D9	Transient receptor potential cation channel subfamily M member 4 splice variant C	PM	3
sp|O43143	Putative pre-mRNA splicing factor RNA helicase	Nuclei	3
sp|O75390	Citrate synthase, mitochondrial precursor	Mitochondria	3
sp|O95169	NADH-ubiquinone oxidoreductase ASHI subunit, mitochondrial precursor	Mitochondria	3

Accession #	Protein Name	Location	# of Peptides
sp|P02383	40S ribosomal protein S26	Ribosome	3
sp|P05388	60S acidic ribosomal protein P0	Ribosome	3
sp|P08574	Cytochrome c1, heme protein, mitochondrial precursor	Mitochondria	3
sp|P10620	Microsomal glutathione S-transferase 1	PM	3
sp|P11233	Ras-related protein Ral-A	PM	3
sp|P16435	NADPH-cytochrome P450 reductase	ER	3
sp|P16991	Nuclease sensitive element binding protein 1	Nuclei	3
sp|P17008	40S ribosomal protein S16	Ribosome	3
sp|P25111	40S ribosomal protein S25	Ribosome	3
sp|P32119	Peroxiredoxin 2	Cytoplasm	3
sp|P36578	60S ribosomal protein L4	Ribosome	3
sp|P46776	60S ribosomal protein L27a	Ribosome	3
sp|P48047	ATP synthase oligomycin sensitivity conferral protein	Mitochondria	3
sp|P50990	T-complex protein 1, theta subunit	Cytoplasm	3
sp|P51151	Ras-related protein Rab-9A	PM	3
sp|P51659	Peroxisomal multifunctional enzyme type 2	Cytoplasm	3
sp|P53985	Monocarboxylate transporter 1	PM	3
sp|P60174	Triosephosphate isomerase	Cytoplasm	3
sp|Q02546	40S ribosomal protein S13	Ribosome	3
sp|Q12931	Heat shock protein 75 kDa, mitochondrial precursor	Mitochondria	3
sp|Q14156	Hypothetical protein KIAA0143	Hypo Memb	3
sp|Q14157	Hypothetical protein KIAA0144	Hypothetical	3
sp|Q15365	Poly(rC)-binding protein 1	Nuclei	3
sp|Q15738	NAD(P)-dependent steroid dehydrogenase	Cytoplasm	3
sp|Q99714	3-hydroxyacyl-CoA dehydrogenase type II	Mitochondria	3
sp|Q9BQE4	Selenoprotein S	PM	3
sp|Q9P0L0	Vesicle-associated membrane protein-associated protein A	PM	3
sp|Q9Y289	Sodium-dependent multivitamin transporter	PM	3
tr|O60625	Endobrevin	PM	3
tr|O94848	Hypothetical protein KIAA0747	Hypo Memb	3
tr|O94963	Hypothetical protein KIAA0887	Hypothetical	3
tr|O95202	Leucine zipper-EF-hand containing transmembrane protein 1	PM	3
tr|O95564	Syntaxin 12 protein	PM	3
tr|Q7Z770	Hypothetical protein	Hypothetical	3
tr|Q8IUW5	Similar to expressed sequence AA536743	Hypo Memb	3
tr|Q8N275	Hypothetical protein FLJ33825	Hypo Memb	3
tr|Q8N2K3	Hypothetical protein FLJ90162	Hypo Memb	3
tr|Q8N4S9	Similar to hypothetical protein	Hypo Memb	3

Accession #	Protein Name	Location	# of Peptides
	FLJ30532		
tr|Q8N5I2	Similar to hypothetical protein CLONE24945	Hypothetical	3
tr|Q8WU20	Suc1-associated neurotrophic factor target	PM	3
tr|Q96EQ4	Similar to hypothetical protein, MNCb-1213	Hypothetical	3
tr|Q96H09	Similar to hypothetical protein FLJ10856	Hypo Memb	3
tr|Q96S30	Hypothetical protein gene +108	Hypo Memb	3
tr|Q9NX40	Hypothetical protein FLJ20455	Hypothetical	3
sp|O75131	Copine III	Cytoplasm	3
sp|O76062	Delta(14)-sterol reductase	PM	3
sp|P00734	Prothrombin precursor	Secreted	3
sp|P07741	Adenine phosphoribosyltransferase	Cytoplasm	3
sp|P17080	GTP-binding nuclear protein RAN	Nuclei	3
sp|P32322	Pyrroline-5-carboxylate reductase	Cytoplasm	3
sp|P40616	ADP-ribosylation factor-like protein 1	PM	3
sp|P52907	F-actin capping protein alpha-1 subunit	Cytoskeleton	3
sp|P53007	Tricarboxylate transport protein, mitochondrial precursor	Mitochondria	3
sp|Q13045	Flightless-I protein homolog	Cytoskeleton	3
tr|Q9NWS8	Hypothetical protein FLJ20627	Hypothetical	3
sp|P02593	Calmodulin	PM	3
sp|P36957	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Mitochondria	3
sp|P49757	Numb protein homolog	PM	3
sp|Q8IWT6	Leucine-rich repeat-containing protein 8 precursor	Hypo Memb	3
sp|Q96S97	Myeloid-associated differentiation marker (SB135)	PM	3
sp|Q9NR30	Nucleolar RNA helicase II	Nuclei	3
tr|Q9BVV7	Hypothetical protein	Hypo Memb	3
sp|P02533	Keratin, type I cytoskeletal 14	Cytoskeleton	3
sp|P06493	Cell division control protein 2 homolog	Nuclei	3
sp|P08779	Keratin, type I cytoskeletal 16	Cytoskeleton	3
sp|P10114	Ras-related protein Rap-2a	PM	3
sp|P13073	Cytochrome c oxidase subunit IV isoform 1, mitochondrial precursor	Mitochondria	3
sp|P16632	40S ribosomal protein S24	Ribosome	3
sp|P17987	T-complex protein 1, alpha subunit	Cytoplasm	3
sp|P30519	Heme oxygenase 2	PM	3
sp|Q15375	Ephrin type-A receptor 7 precursor	PM	3
sp|Q9NR31	GTP-binding protein SAR1a	PM	3
sp|Q9Y6B6	GTP-binding protein SAR1b	PM	3
tr|Q86X10	Hypothetical protein KIAA1219	Hypothetical	3
sp|P43250	G protein-coupled receptor kinase GRK6	PM	3

Accession #	Protein Name	Location	# of Peptides
tr|O75050	Hypothetical protein KIAA0462	Hypothetical	3
tr|Q7Z794	Keratin 1b	Cytoskeleton	3
tr|Q8NBP9	Hypothetical protein NT2RP1001023	Hypo Memb	3
sp|O94826	Mitochondrial precursor proteins import receptor	Mitochondria	3
sp|P15822	Zinc finger protein 40	Nuclei	3
sp|P18433	Protein-tyrosine phosphatase alpha precursor	PM	3
sp|P22455	Fibroblast growth factor receptor 4 precursor	PM	3
sp|Q02543	60S ribosomal protein L18a	Ribosome	3
sp|Q02877	60S ribosomal protein L26	Ribosome	3
sp|Q9BVK6	Glycoprotein 25L2 precursor	ER	3
tr|Q86UK0	ABCA12 transporter subfamily A	PM	3
sp|O14493	Claudin-4	PM	2
sp|O15126	Secretory carrier-associated membrane protein 1	PM	2
sp|O15269	Serine palmitoyltransferase 1	ER	2
sp|O43752	Syntaxin 6	PM	2
sp|O96000	NADH-ubiquinone oxidoreductase PDSW subunit	Mitochondria	2
sp|O96008	Probable mitochondrial import receptor subunit TOM40 homolog	Mitochondria	2
sp|P00533	Epidermal growth factor receptor precursor	PM	2
sp|P00966	Argininosuccinate synthase	Cytoplasm	2
sp|P02248	Ubiquitin	Cytoplasm	2
sp|P04004	Vitronectin precursor	Secreted	2
sp|P07478	Trypsin II precursor	Secreted	2
sp|P07737	Profilin I	Cytoskeleton	2
sp|P10515	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Mitochondria	2
sp|P11234	Ras-related protein Ral-B	PM	2
sp|P12280	Microsomal signal peptidase 23 kDa subunit	ER	2
sp|P13641	Small nuclear ribonucleoprotein Sm D1	Nuclei	2
sp|P14625	Endoplasmin precursor	ER	2
sp|P17858	6-phosphofructokinase, liver type	Cytoplasm	2
sp|P19367	Hexokinase, type I	Mitochondria	2
sp|P20674	Cytochrome c oxidase polypeptide Va, mitochondrial precursor	Mitochondria	2
sp|P21860	Receptor protein-tyrosine kinase erbB-3 precursor	PM	2
sp|P22695	Ubiquinol-cytochrome C reductase complex core protein 2	Mitochondria	2
sp|P23526	Adenosylhomocysteinase	Cytoplasm	2
sp|P25120	60S ribosomal protein L8	Ribosome	2
sp|P31943	Heterogeneous nuclear	Nuclei	2

Accession #	Protein Name	Location	# of Peptides
	ribonucleoprotein H		
sp|P35222	Beta-catenin	Cytoplasm	2
sp|P36542	ATP synthase gamma chain, mitochondrial precursor.	Mitochondria	2
sp|P38646	Stress-70 protein, mitochondrial precursor	Mitochondria	2
sp|P39026	60S ribosomal protein L11	Ribosome	2
sp|P39030	60S ribosomal protein L15	Ribosome	2
sp|P40926	Malate dehydrogenase, mitochondrial precursor	Mitochondria	2
sp|P43307	Translocon-associated protein, alpha subunit	ER	2
sp|P48643	T-complex protein 1, epsilon subunit	Cytoplasm	2
sp|P49768	Presenilin 1	PM	2
sp|P50402	Emerin	Nuclei	2
sp|P50416	Carnitine O-palmitoyltransferase I, mitochondrial liver isoform	Mitochondria	2
sp|P51809	Synaptobrevin-like protein 1	PM	2
sp|P57721	Poly(rC)-binding protein 3	Cytoplasm	2
sp|P80723	Brain acid soluble protein 1	PM	2
sp|Q04941	Intestinal membrane A4 protein	ER	2
sp|Q07812	Apoptosis regulator BAX, membrane isoform alpha	Mitochondria	2
sp|Q10589	Bone marrow stromal antigen 2	PM	2
sp|Q14108	Lysosome membrane protein II	PM	2
sp|Q15223	Poliovirus receptor related protein 1 precursor	PM	2
sp|Q15363	Cop-coated vesicle membrane protein p24 precursor	PM	2
sp|Q15366	Poly(rC)-binding protein 2	Nuclei	2
sp|Q15382	GTP-binding protein Rheb	PM	2
sp|Q16563	Pantophysin	PM	2
sp|Q96HY6	Protein C20orf116	Secreted	2
sp|Q9C0B5	Zinc finger DHHC domain containing protein 5	Membrane	2
sp|Q9GZN8	Protein C20orf27	Hypothetical	2
sp|Q9H223	EH-domain containing protein 4	Cytoplasm	2
sp|Q9NRW7	Vacuolar protein sorting-associated protein 45	Golgi	2
sp|Q9NUE0	Zinc finger DHHC domain containing protein 18	Membrane	2
sp|Q9NY35	Protein C3orf4	Membrane	2
sp|Q9Y282	Serologically defined breast cancer antigen NY-BR-84	Membrane	2
sp|Q9Y3E5	Protein CGI-147	Mitochondria	2
tr|O75545	Hypothetical protein	Hypo Memb	2
tr|O94843	Hypothetical protein KIAA0739	Hypo Memb	2
tr|O94918	Hypothetical protein KIAA0829	Hypothetical	2
tr|O95164	Hypothetical protein	Hypothetical	2
tr|O95297	ZERO related protein	PM	2

Accession #	Protein Name	Location	# of Peptides
tr|Q86UE4	LYRIC/3D3	Hypo Memb	2
tr|Q8NC56	Hypothetical protein FLJ90478	Hypo Memb	2
tr|Q8NC61	Hypothetical protein FLJ90468	Hypo Memb	2
tr|Q8NCL5	Hypotheticalprotein FLJ90173	Hypo Memb	2
tr|Q8WVX9	Similar to RIKEN cDNA 3732409C05 gene	Hypothetical	2
tr|Q96AZ5	Similar to hypothetical protein DKFZp566A1524	Hypothetical	2
tr|Q9BR02	Novel protein similar to RPL23A	Ribosome	2
tr|Q9BTZ3	Hypothetical protein	Hypothetical	2
tr|Q9BUB7	Similar to hypothetical protein FLJ20533	Hypo Memb	2
tr|Q9NWT0	Hypothetical protein	Hypothetical	2
tr|Q9Y2G0	Hypothetical protein KIAA0953	Hypothetical	2
sp|O14925	Mitochondrial import inner membrane translocase subunit TIM23	Mitochondria	2
sp|P00387	NADH-cytochrome b5 reductase	ER	2
sp|P11518	60S ribosomal protein L7a	Ribosome	2
sp|P13164	Interferon-induced transmembrane protein 1	PM	2
sp|P13987	CD59 glycoprotein precursor	PM	2
sp|P59998	ARP2	Cytoskeleton	2
sp|Q15262	Receptor-type protein-tyrosine phosphatase kappa precursor	PM	2
tr|Q7Z3D5	Hypothetical protein DKFZp686A1586	Hypo Memb	2
tr|Q8N138	Hypothetical protein FLJ90330	Hypo Memb	2
tr|Q9GZT3	Hypothetical protein PD04872	Hypothetical	2
sp|O43169	Cytochrome b5 outer mitochondrial membrane isoform precursor	Mitochondria	2
sp|O75897	Sulfotransferase 1C2	Cytoplasm	2
sp|O75964	ATP synthase g chain, mitochondrial	Mitochondria	2
sp|P32969	60S ribosomal protein L9	Ribosome	2
sp|P46977	Oligosaccharyl transferase STT3 subunit homolog (B5)	PM	2
sp|P56134	ATP synthase f chain, mitochondrial	Mitochondria	2
sp|Q13835	Plakophilin 1	Cytoskeleton	2
sp|Q14202	Zinc finger protein 261	Nuclei	2
sp|Q96AX2	Ras-related protein Rab-37	PM	2
sp|Q9Y446	Plakophilin 3	Cytoskeleton	2
tr|Q86YS6	RAB11B	PM	2
tr|Q9NZ23	Gastric-associated differentially-expressed protein YA61P	Cytoplasm	2
sp|O43175	D-3-phosphoglycerate dehydrogenase	Cytoplasm	2
sp|O43707	Alpha-actinin 4	Cytoskeleton	2
sp|O75340	Programmed cell death protein 6	Cytoplasm	2
sp|P01270	Parathyroid hormone precursor	Secreted	2
sp|P02647	Apolipoprotein A-I precursor	Secreted	2
sp|P07237	Protein disulfide isomerase precursor	ER	2
sp|P10412	Histone H1.4	Nuclei	2

Accession #	Protein Name	Location	# of Peptides
sp|P19634	Sodium/hydrogen exchanger 1	PM	2
sp|P21181	Cell division control protein 42 homolog	PM	2
sp|P54764	Ephrin type-A receptor 4 precursor	PM	2
sp|Q13277	Syntaxin 3	PM	2
tr|O14787	Transportin 2	Nuclei	2
tr|Q8N789	Hypothetical protein FLJ25911	Hypo Memb	2
tr|Q9P0N6	Hypothetical protein	Hypo Memb	2
sp|P39027	40S ribosomal protein S15a	Ribosome	2
tr|Q15290	RB protein binding protein	Cytoplasm	2
tr|Q7RTP1	Hypothetical protein	Hypothetical	2
tr|Q7Z392	Hypothetical protein DKFZp686B15150	Hypothetical	2
tr|Q96F25	Similar to RIKEN cDNA 5430428G01 gene	Hypo Memb	2
tr|Q9H652	Hypothetical protein FLJ22603	Hypo Memb	2
tr|Q9H6C8	Hypothetical protein FLJ22389	Hypothetical	2
tr|Q9HCF4	Hypothetical protein KIAA1618	Hypothetical	2
tr|O60350	Sodium bicarbonate cotransporter2	Membrane	2
sp|P78504	Jagged 1 precursor	PM	2

Table 5: List of protein identified from the basolateral plasma membrane fraction isolated from MXR MCF-7 cells. The SwissProt and Trembl accession numbers are indicated for each protein. Subcellular Location Abbreviations: PM – plasma membrane; Hypo Memb – hypothetical membrane protein; Hypothetical - hypothetical protein; ER – endoplasmic reticulum; Membrane – membrane protein of unspecified location.
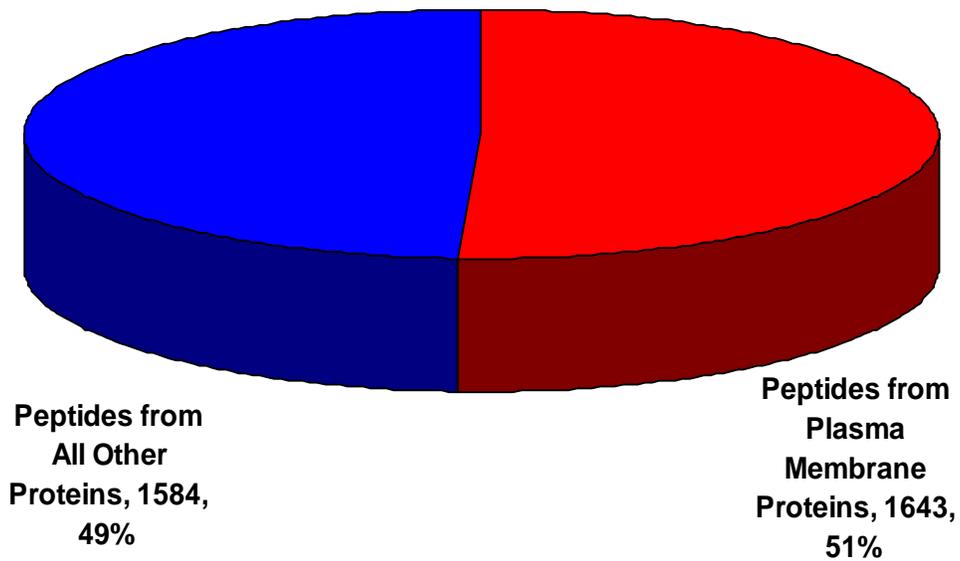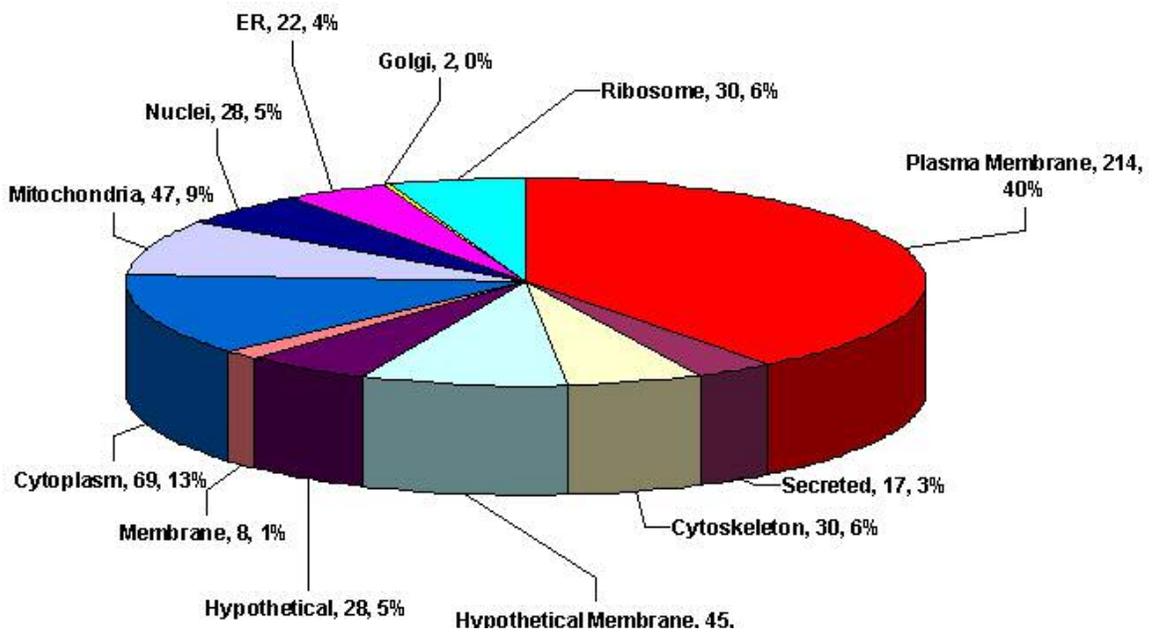
Figure 25: A. Subcellular distribution of proteins identified in the MXR MCF-7 basolateral plasma membrane fraction. B. The percentage of peptides used for these protein identifications that originated from plasma membrane proteins.
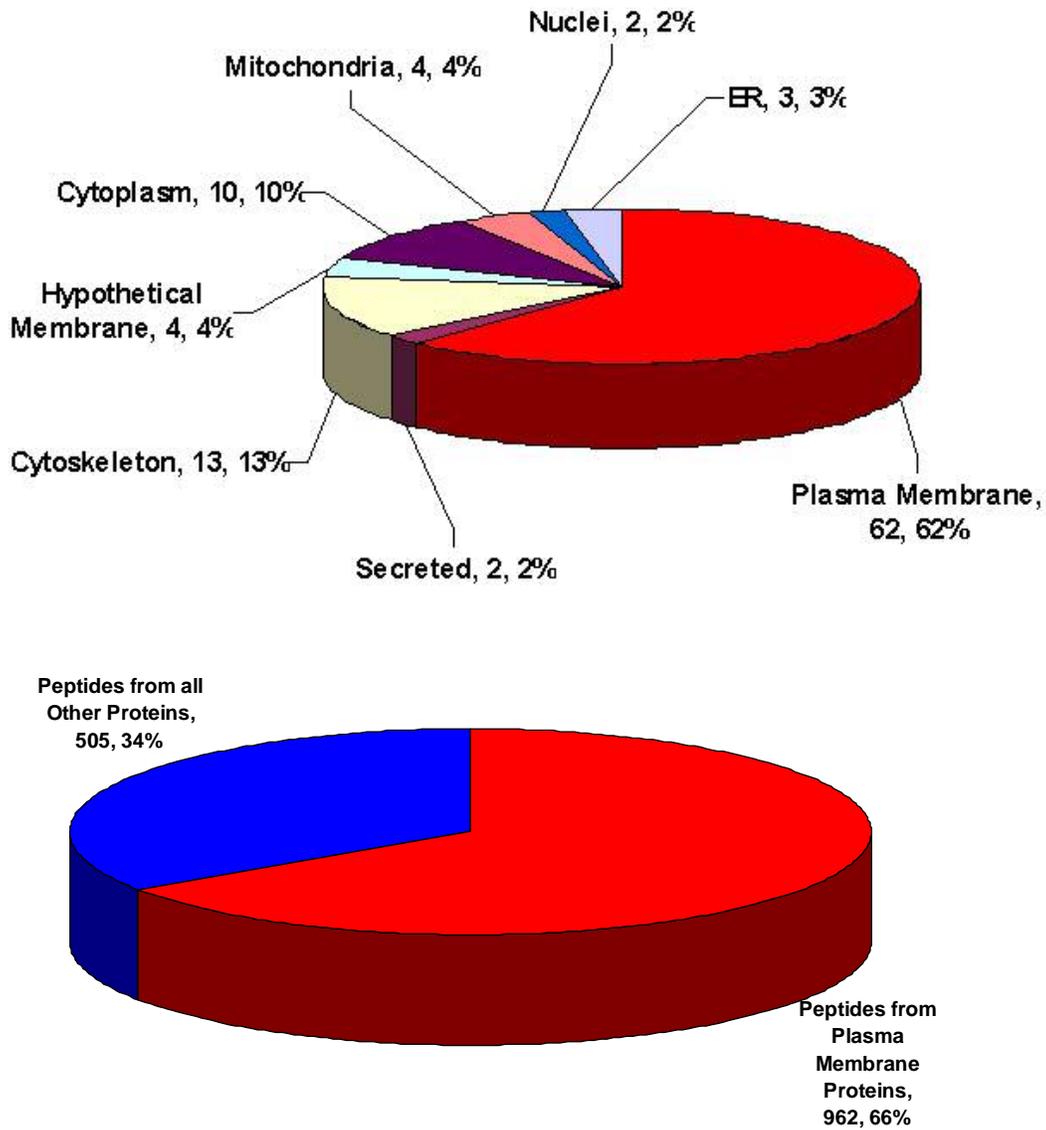
Figure 26: A. Subcellular distribution of the 100 proteins most reliably identified in the MXR MCF-7 basolateral plasma membrane fraction based, on the number of peptides used to make the identifications and their scores. B. The percentage of peptides used for these top 100 protein identifications which originated from plasma membrane proteins.

Membrane proteins represent a subset of proteins that are harder to analyze than other proteins. Isolation, separation, and digestion are all problematic, especially with subcellular fractions such as the plasma membrane which make up so little of the total cell content. Designing strategies for the proteomic analysis of membrane subfractions often requires non-traditional techniques and procedures.

Recently there have been some advances in the techniques and procedures of 2DGE for the separation of complex protein mixtures, including hydrophobic and membrane proteins.[62] Although 2DGE can be modified or optimized to resolve and separate specific membrane proteins, there does not seem to be one technique that works universally for them and so 2DGE is usually excluded as a viable option.[38; 39; 58] The chemical cleavage agent CNBr has had some success in the cleavage of membrane proteins in situ and has also been used in combination with trypsin to increase the efficiency of protein digestion. Unfortunately CNBr is dangerous to work with and many scientists have tried to find alternatives to using this substance.[57]

The strength of the method we developed for the proteomic analysis of plasma membrane is the improvement in the isolation method. This improved enrichment allows for the use of SDS-PAGE for the separation of the proteins.[48] Although this technique has a much lower resolution than 2DGE, it provides a rudimentary separation of membrane proteins by their molecular weight. Once the proteins are separated, the gel then provides an environment in which the proteins can be digested. The real separation is performed in the LC-MS experiments

after the peptides are extracted. This method can also provide excellent sequence coverage of the membrane proteins as is shown in **Figures 27** and **28**.

Of the 540 proteins identified, 214, or 40% of these, have been previously categorized as plasma membrane proteins in the literature (**Figure 25**). There were also many plasma membrane associated proteins identified, such as secreted proteins associated with the exterior of the plasma membrane (17 proteins, 3%), or cytoskeletal structural proteins (30 proteins, 6%) which are strongly associated with the interior of the plasma membrane. There were also 73 proteins identified that had not been previously identified or reported, and were identified using predicted amino acid sequences from human genes. Of these hypothetical proteins, 45 were predicted to have transmembrane spanning regions according to the TMHMM program which is used to predict transmembrane helices in proteins.[63] There was also a small subset of proteins that are classified as "Membrane" which were categorized as membrane proteins in the literature, but with no subcellular location defined. As shown in **Figure 26**, of the top 100 proteins identified, based on the confidence scores and the number of peptides identified from each protein, the majority are from the plasma membrane.

Reset to Default... Set as Default Digest Un-Map Map

Description: | 4F2 CELL-SURFACE ANTIGEN HEAVY CHAIN (4F2HC) (LYMPHOCYTE AC | 317 of 529 matched - 59.9%

Sequence mapped to:

$C_{2600}H_{4089}N_{697}O_{780}S_{11}$ Mono MW: 57908.8653   Revert to Saved

Amino Acid Comp.   Selection: 1   Avg MW: 57944.4899   Find...

A	50	9.5%
C	2	0.4%
D	32	6.0%
E	33	6.2%
F	21	4.0%
G	43	8.1%
H	8	1.5%
I	15	2.8%
K	28	5.3%
L	82	15.5%
M	9	1.7%
N	17	3.2%
P	24	4.5%
Q	25	4.7%
R	23	4.3%
S	48	9.1%
T	21	4.0%
V	26	4.9%
W	13	2.5%
X	0	0.0%
Y	9	1.7%

```
MSQDTEVDMK EVELNELEPE KQPMNAASGA AMSLAGAEKN GLVKIKVAED EAEAAAAAKF   60
TGLSKEELLK VAGSPGWVRT RWALLLLFWL GWLGMLAGAV VIIVRAPRCR ELPAQKWWHT  120
GALYRIGDLQ AFQGHGAGNL AGLKGRLDYL SSLKVKGLVL GPIHKNQKDD VAQTDLLQID  180
PNFGSKEDFD SLLQSAKKKS IRVILDLTPN YRGENSWFST QVDTVATKVK DALEFWLQAG  240
VDGFQVRDIE NLKDASSFLA EWQNITKGFS EDRLLIAGTN SSDLQQILSL LESNKDLLLT  300
SSYLSDSGST GEHTKSLVTQ YLNATGNRWC SWSLSQARLL TSFLPAQLLR LYQLMLFTLP  360
GTPVFSYGDE IGLDAAALPG QPMEAPVMLW DESSFPDIPG AVSANMTVKG QSEDPGSLLS  420
LFRRLSDQRS KERSLLHGDF HAFSAGPGLF SYIRHWDQNE RFLVVLNFGD VGLSAGLQAS  480
DLPASASLPA KADLLLSTQP GREEGSPLEL ERLKLEPHEG LLLRFPYAA              529
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Figure 27: The total sequence coverage of the 4F2 cell surface antigen is 59.9%.

Figure 28: The total sequence coverage of the transferring receptor protein 1 is 41.4%.

The protein assignments we use to determine subcellular location were based on the information from the databases. Other researchers have reported a higher percentage of plasma proteins identified from their isolation, using the Biotin Directed Affinity Purification (BDAP) plasma membrane isolation method followed by the same technique of SDS-PAGE, in-gel digestion and LC-MS of the recovered peptides.[51] A closer look at the supplementary results provided by the authors show that they assign every membrane protein they encounter as a plasma membrane protein, even proteins found exclusively in other organelles such as the ATP synthase protein complex found in the mitochondria. Using their criteria, our method would yield a much higher enrichment then reported here, but assignments would not be correct.

Because we provide no experimental results for the assignment of protein subcellular locations, we adhere strictly to protein locations reported in the literature. This, combined with our requirement for 2 high scoring peptides/protein for identifications, and our subsequent manual sequencing provides a very low probability for false positives or false assignments of plasma membrane proteins.

Because the plasma membrane sample is highly enriched in relation to the whole cell lysate, the low resolution separation technique of SDS-PAGE is sufficiently effective to permit the identification of so many plasma membrane proteins. Since we are isolating the plasma membranes as large sheets with the plasma membrane proteins still embedded, we do not have to worry about losing protein due to precipitation in protein solubilization steps during the isolation itself. Once the plasma membranes

are isolated and washed, they are solubilized directly in SDS solution, followed by SDS-PAGE analysis, and this offers minimal opportunity for protein loss.

Protein and peptide sequencing, along with the antibody analysis, suggest that the plasma membrane isolation technique is quite effective, and we estimate that of the total protein loaded on the gels, over 50% of proteins by mass originate from the plasma membrane. Estimating that the plasma membrane makes up about 2-3% of the cell mass, and assuming a 20 fold enrichment, the estimated amount of plasma membrane protein loaded in the SDS-PAGE experiment would be 40-60% of the total protein loaded. This figure, along with the fact that 51% of the peptides sequenced originated from plasma membrane proteins, agrees with our estimation that over 50% of our isolated proteins originate from the plasma membrane.

By performing online nanoLC coupled with mass spectrometry we were able to obtain many more protein identifications than by using static infusion nanospray. Each of the gel slices contained many proteins so when the peptides were analyzed using static infusion nanospray, many of the less abundant peptides that were of similar mass to some of the more abundant peptides were not observed. Adding the chromatographic separation step allowed the complex peptide mixture to be separated and introduced into the mass spectrometer at a slower pace instead of all at once. This allowed for the identification of many of the lesser abundant peptides which would not normally be observed.

Not all of the peptides from the proteins identified are observed during a proteomic analysis.[64] Peptides containing post translational modifications are more difficult to identify then their unmodified counterparts. Identifying these peptides is often tedious and typically in a quantitative or qualitative proteomic analysis, these peptides which contain post-translational modifications are overlooked.[65] This is because 100% sequence coverage is not necessary for the identification or relative quantitation of proteins in different samples.[66; 67; 68; 69; 70]

Another reason 100% sequence coverage of proteins is not obtained during proteomic analysis is that not all peptides get picked for tandem mass spectrometry during an LC-MS experiment. These experiments tend to pick ions with the highest signal intensities and if there are many proteins eluting from the column and entering the mass spectrometer at the same time, ions with lower intensities from peptides abundant in lower levels or from peptides which do not ionize well will often be overlooked.[71]

As was stated previously, we estimate that greater than 50% of the total protein loaded onto the SDS-PAGE gels are plasma membrane proteins. Enrichment levels of greater than 50% are difficult to obtain when enriching isolating plasma membranes. Reasons for this could be that upon cell lysis, membrane components mix with one another, thereby increasing the number of membrane proteins identified which are not associated with the plasma membrane such as proteins from the ER or Golgi complex. Another possible reason for the lower enrichment levels of plasma membranes are

that membranes form small vesicles and can capture many of the cytoplasmic proteins

present during cell lysis. These soluble proteins are then identified and analyzed

along with the membrane proteins and contribute to the lower enrichment levels.

Chapter 4: Comparison of Plasma Membrane Proteins from Drug Resistant and Drug Susceptible MCF-7 Breast Cancer Cell Lines

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A. Comparative Proteomics Techniques

In the previous chapter, we designed a qualitative "bottom-up" proteomic analysis for the analysis of plasma membrane proteins from cell cultures. While this information is useful for identifying what proteins are present in a particular sample, it does not give information on the protein abundance levels of the individual proteins identified. Comparative proteomic experiments can be used to obtain information about the relative abundance levels of proteins from cells or tissues in different states, such as diseased and healthy, treated and untreated, or from different samples altogether. This chapter will outline a method developed for comparing the differences in protein abundances from proteins from a mitoxantrone resistant (MXR) MCF-7 cell line and from a mitoxantrone susceptible, non-resistant parental cell line.

Historically, the first comparative experiments were performed using 2DGE. Initially, proteins from 2 different samples would be separated on 2D gels. The 2D gel arrays are scanned and the images compared by differential densitometry. Protein abundance changes are observes as increased or decreases in gel spot densities.[49; 72; 73]

Reproducibility among 2D gels from samples originating from the same source is usually reasonable and has been improved over time, but becomes more problematic when performing comparative studies. Small differences in the way proteins are separated on 2D gels can be interpreted as changes in protein abundances in comparative studies. This can usually be overcome by running multiple gels and averaging the scanned images together. These averaged images can then be compared to the averaged images from another sample and these averages compared.[74] The results from this type of experiment can be dramatic and differences in protein abundances can be quickly ascertained.

A common misconception in the analysis of mass spectrometry data is that there exists a relation between the peak intensities and the concentrations of different peptides. This is not the case. The ionization efficiencies of peptides differ from one peptide to another and are dependent upon the primary structure of individual peptides and the chemical properties of the amino acid side chains which make up these peptides. Therefore there is no relation between the peptide signal intensities from differing peptides.[71] That having been said, there is a correlation between the peptide signal intensities and the concentrations of identical peptides.

Recently, alternatives to the gel-based method of comparative analysis have been developed using chemical labeling using stable isotopes such as ^{2}H, ^{13}C, ^{15}N, and ^{18}O.[72; 75; 76; 77] Chemical species made up of these stable isotopes contain the same

chemical properties as their lighter counterparts but have an increased mass. Therefore, if separate peptide mixtures are chemically modified with either light or heavy labels, the relative intensities and isotope ratios of each of these species can be determined and since the peptides have the same ionization efficiencies, the relative abundances of these peptides and therefore the concentrations of the proteins they originate from can be measured.

In this study we introduced isotope labels by metabolic labeling, which labels proteins during cell growth. Cell cultures are grown in cell culture media containing $^{13}C_6$ labeled arginine and lysine residues as the predominant source of these amino acids (**Figure 29**). These amino acids are then incorporated into all newly synthesized proteins and all tryptic peptides containing 1 labeled amino acid will have masses of 6 Da higher then their unlabeled counterparts. A comparative proteomic analysis of proteins from two different cell lines can therefore be performed by growing one cell line in normal growth conditions, and growing the other cell line in the cell culture media containing the labeled amino acids. The cells can then be harvested and the proteins isolated, followed by analysis using mass spectrometry. Using this metabolic labeling technique we labeled the proteins from a drug susceptible MCF-7 parental cell line using the metabolic labeling technique described here and compared the protein abundance changes between it and an MCF-7 cell line selected for resistance to mitoxantrone (MXR MCF-7).

The role of plasma membrane proteins in drug resistance has been previously studied in the mitoxantrone resistant MCF-7 breast cancer cell line.[78; 79] It has been shown that this cell line does not overexpress P-glycoprotein or any of the proteins from the multidrug resistance associated protein family, which are the plasma membrane proteins usually associated with multidrug resistance, when compared to the parental drug susceptible MCF-7 cell line.[80; 81] mRNA analysis and Western immunoassay experiments have shown that this cell line overexpresses the ATP-binding cassette protein ABCG2 commonly known as breast cancer resistance protein (BCRP), and the mechanisms of drug resistance used by this cell line has been shown to be independent of the P-glycoprotein and multidrug resistance associated protein mediated drug resistance pathways.[78; 81; 82]
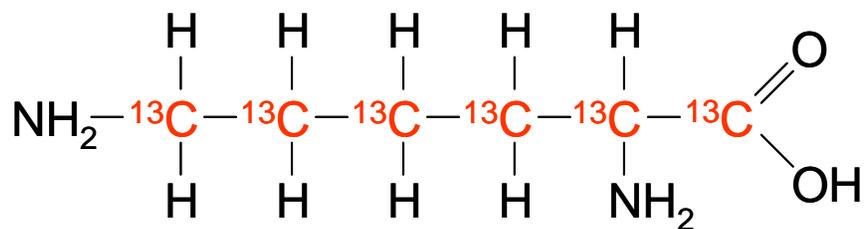
¹³C Labeled Lysine
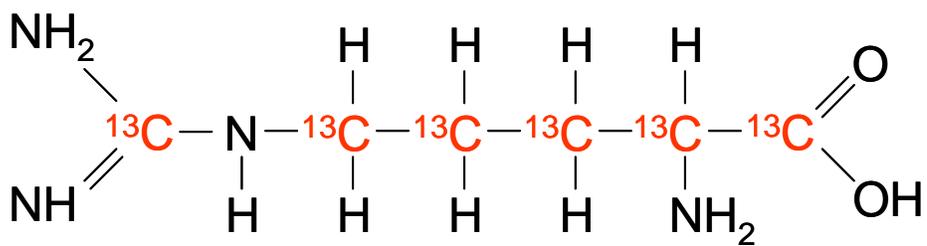
¹³C Labeled Arginine

Figure 29: Structure of the 13C labeled amino acids arginine and lysine

B. Materials and Methods

Materials

The Criterion precast gel system, Criterion precast gels (13.3 x 8.7 cm 4-15%), Biosafe Coomassie stain, 10x Tris/Glycine/SDS Buffer, pre-stained protein broad range standards, and Laemmli sample buffer were purchased from Bio-Rad (Hercules, CA) . DTT, iodoacetamide, fetal calf serum, Penicillin-Streptomycin solution, and TFA were purchased from Sigma Aldrich (St. Louis, MO). NH_4HCO_3, formic acid, acetonitrile, acetic acid, methanol, and $CaCl_2$ were purchased from Fisher (Pittsburg, PA). Modified porcine trypsin was purchased from Promega (Madison, WI). Improved Minimal Essential Medium was purchased from American Type Culture Collection (Manassas, VA). MEM (Eagle) Media with Earle's Salts without L-lysine or L-arginine, and Dialyzed Fetal Bovine Serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Carbon-13 (^{13}C) labeled L-lysine and L-arginine isotopes at 98% purity was purchased from Cambridge Isotope Laboratories (Andover, MA).

Equipment

Mass spectra were obtained using an Applied Biosystems Qstar Pulsar i (Foster City, CA) with a nanospray ion source from Protana (Odense, Denmark). NanoLC-MS was performed online with the LC Packings Ultimate Nano LC System (Sunnyvale, CA).

LC-MS head with Liquid Junction were purchased from Proxeon (Odense, Denmark). Nanospray emitters were purchased from New Objectives (Woburn, MA).

Metabolic labeling of the MCF-7 Breast Cancer Cell Line

Metabolically labeled drug susceptible MCF-7 cells were grown using specially prepared media. Isotopically labeled L-lysine and L-arginine amino acids were added to Minimal Essential Medium lacking the amino acids L-lysine and L-arginine, so that the predominant source of biologically usable lysine and arginine are labeled with the ^{13}C isotopes. The fetal bovine serum used was made from 25% of the normal serum and 75% of the dialyzed serum to minimize the introduction of unlabeled lysine and arginine. The labeled MCF-7 cells were grown to confluence using the same protocol and growth conditions as the MXR MCF-7 cells, but using the modified media and fetal bovine serum specified in place of the normal recipes. Unlabeled MXR MCF-7 cells were grown using the same conditions as the labeled cells but using unlabeled L-arginine and L-lysine in the cell culture media.

One Dimensional SDS-PAGE Analysis

Twenty µg of plasma membrane protein from MXR MCF-7 cells and twenty µg of plasma membrane protein from metabolically labeled drug susceptible MCF-7 cells were mixed together and loaded onto 4-15% gels and run according to manufacturer's specifications using the Bio-Rad Criterion precast gel system. Following

electrophoresis, the gels were stained using Bio-Rad Biosafe Coomassie stain and the stained gels were scanned using a GS-800 densitometer from Bio-Rad (Hercules, CA) .

Mass Spectrometry

The SDS-PAGE gel was excised into 28 gel bands and tryptic digestion was performed on the gel slices as before.[61] After extraction from the gel bands, the tryptic peptides were placed in 0.1% formic acid (FA) in preparation for nanoLC-MS/MS analysis. The tryptic peptides were analyzed by online nanoLC-MS/MS. Reverse phase conditions were A: 97.5% H_2O/2.5% ACN/0.1%FA, B: 97.5% ACN/2.5% H_2O/0.1%FA with a 60 minute gradient from 5%-35%B on a PepMap 75µm I.D., 15cm, 3µm, 100Å column from LC Packings (Sunnyvale, CA).

Protein Identification

Each protein was identified based on sequences from two or more peptides using the integrated Qstar software Analyst QS with Bioanalyst and ProID with a minimum confidence level of 99.4% and were also manually sequenced from the tandem mass spectrometry data.

Protein Quantitation

Ratios of unlabeled/labeled monoisotopic peak areas were calculated to determine protein abundance changes between the two cell lines. Corrections were made in the final ratios to compensate for the extent of incorporation determined for the label (90%). Ratios ≥ 2 or ≤ 0.5, which correspond to a 100% abundance difference between the 2 cell lines, were considered biologically significant and subsequently reported. Replicate experiments were performed using mixtures from 4 different plasma membrane protein isolations from both unlabeled MXR MCF-7 cells and labeled drug susceptible MCF-7 cells.

C. Results and Discussion

The technique of metabolic stable isotope labeling involves the incorporation of stable isotopes into cells during cell growth so that all newly synthesized biomolecules incorporate these isotopes into their chemical structure. Initially this was done with single celled organisms such as yeast and bacteria but more recently this technique has been successful with cell culture and multicellular organisms such as *Caenorhabditis elegans*, and *Drosophila melanogaster* [83; 84; 85; 86; 87; 88]

Our lab has developed a technique for the labeling of proteins with both ^{13}C arginine and ^{13}C lysine.[72] Standard growth conditions for mammalian cell culture uses 90% cell culture media and 10% fetal bovine serum. Commercially available cell culture media deficient in the amino acids arginine and lysine was mixed with labeled ^{13}C arginine and lysine to ensure that the only source of these amino acids is the ^{13}C variety. In the initial growth conditions used, dialyzed fetal bovine serum was used to minimize the addition of unlabeled ^{12}C arginine and lysine from other proteins or peptides in the serum. These conditions resulted in poor cell growth and it was subsequently found that cells would grow normally by using 2.5% of the standard fetal bovine serum with 7.5% of the dialyzed serum. It is hypothesized that the serum contains growth factors necessary for normal growth of these estrogen-positive cells, and that the dialyzed serum was most probably deficient in these factors. The
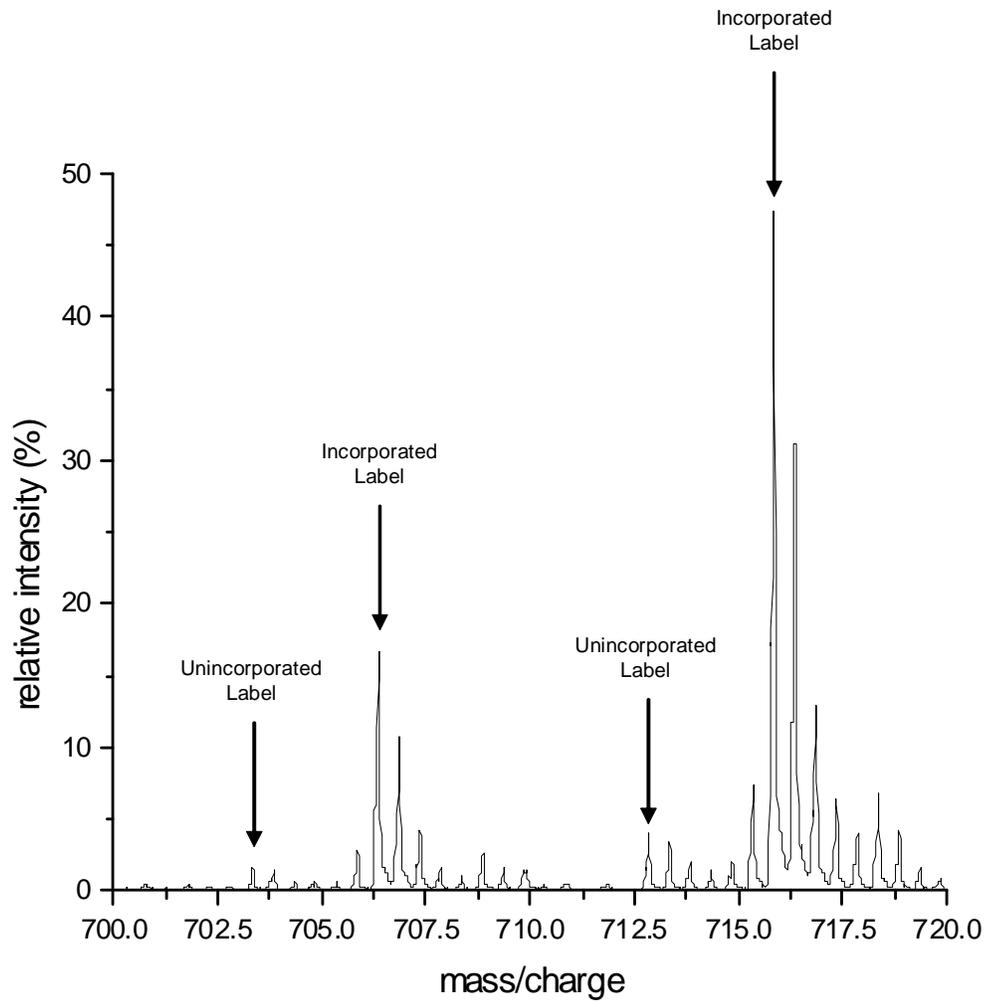
Figure 30: 90% incorporation of the labeled amino acids is achieved in the drug susceptible MCF-7 breast cancer cell line using the metabolic labeling technique developed.

percentage of incorporation of the labeled amino acids into all newly synthesized proteins was determined by the analysis of the mass spectra of the labeled proteins alone, along with 1:1 mixtures of labeled and unlabeled proteins from the same MCF-7 drug susceptible cell line.[72] Using this modified recipe, we were able to metabolically label the drug susceptible MCF-7 parental cell line with a 90% incorporation rate of the ^{13}C amino acids (**Figure 30**).

The objective of this portion of the study was to determine the differences in the abundance levels of proteins from the plasma membranes of mitoxantrone drug resistant and drug susceptible MCF-7 cells. Plasma membranes from the two cell lines were isolated and the proteins extracted as described in the previous section. The protein concentrations of these fractions were determined and the plasma membrane proteins from the 2 samples were mixed at a 1:1 ratio.

SDS-PAGE separation of the protein mixture was performed and the gel was stained and excised into 28 gel bands. The gel bands were subjected to in-gel tryptic digestion and the peptides were recovered and analyzed by LC-MS. Peptide pairs resulting from the in-gel digestions co-eluted and protein quantitation was performed using the data obtained. **Figure 31** shows a scheme for the procedure used for this analysis.

LC-MS was performed on the peptides extracted from the SDS-PAGE gel bands. The peptides co-eluted from the reverse phase column and the area under the

monoisotopic peaks from the mass spectra of matching labeled and unlabeled peptides were compared to each other to determine differences in relative abundances of the proteins from the two cell lines. **Figure 32** shows an LC-MS survey scan of peptides extracted from band 22.
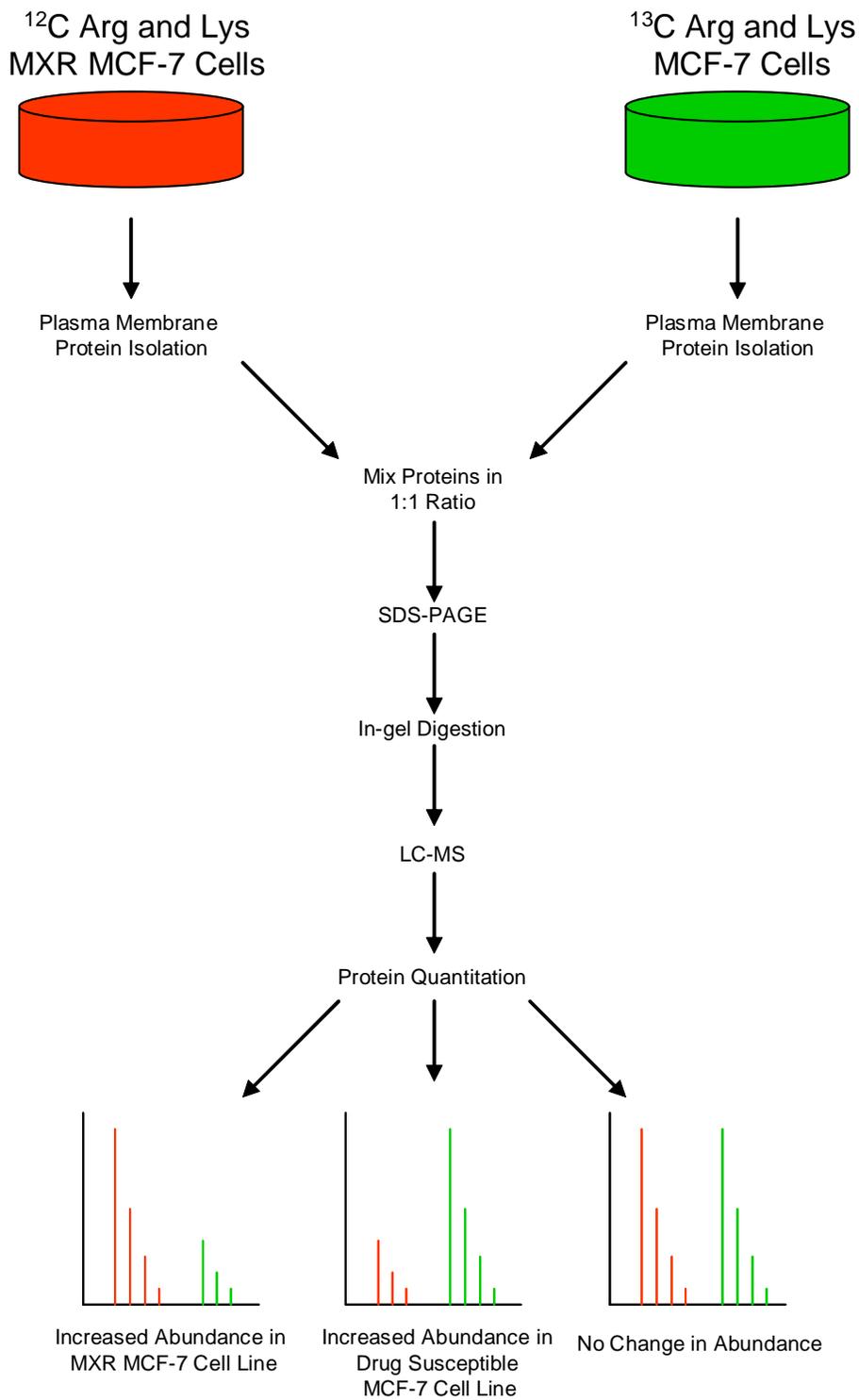
Figure 31: Scheme for the quantitation of protein from the plasma membranes from a MXR MCF-7 cell line and a drug susceptible MCF-7 cell line.
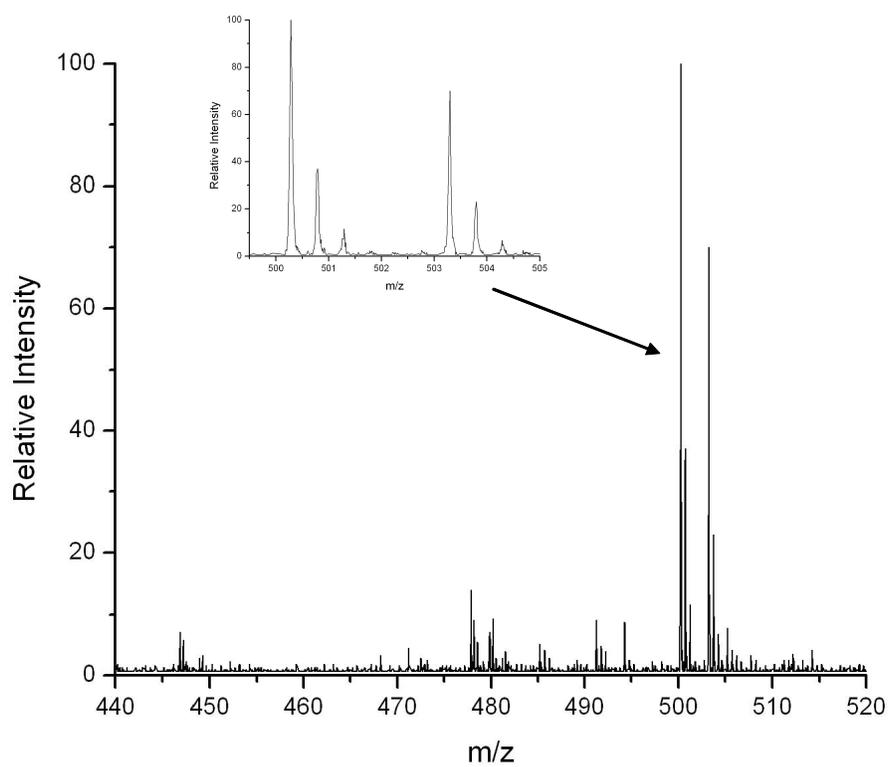
Figure 32: Labeled and unlabeled peptide pairs extracted from band 22 and analyzed by LC-MS. The inset shows an expanded view of the doubly charged peptide AGTQLLAGLR from the plasma membrane protein Integrin alpha-V.

Pairs of labeled and unlabeled peptides were compared for all proteins identified. Abundance ratios were determined and the proteins for which changes in abundance were more than 100% were reported. Multiple peptides were used for protein identification and for calculations for the abundance changes.

Figure 33 shows the labeled and unlabeled peptide pairs used to calculate the relative abundances of the 4F2 cell-surface antigen from susceptible and drug resistantMCF-7 cells. The ratio was calculated for each individual peptide pair and the values are averaged in the final results. **Table 6** shows the experimental data used to make these calculations for the quantitation of the 4F2 cell-surface antigen, a known amino acid transporter.

Changes in the ratios of the intensities of unlabeled/labeled peptides >2 or <0.5 are considered significant. **Table 7** lists all of the proteins whose relative abundance changes were over 100%. The proteins whose names are in red have higher abundances in the MXR MCR-7 cell line, while proteins listed in blue have higher abundances in the drug susceptible MCR-7 parental cell line. **Figure 34** is a graph summarizing the relative abundance changes of the proteins listed in **Table 7**.

All of the proteins showing abundance changes have been previously identified in the literature except for the Hypothetical protein DKFZp686D0452. This protein is predicted to be in the integrin family of proteins and has 96% sequence homology
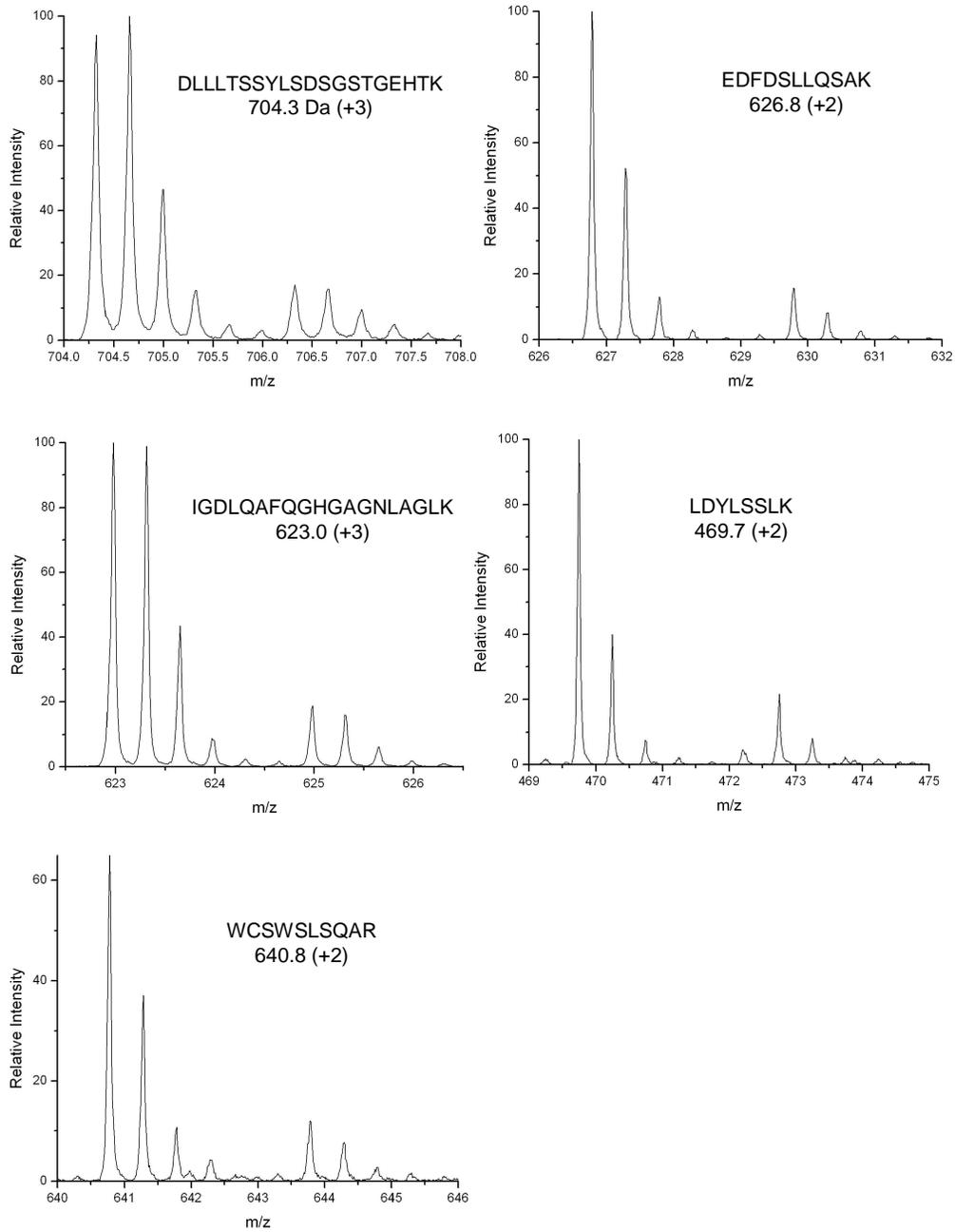
Figure 33: Peptide pairs from the 4F2 cell surface antigen protein analyzed from the labeling experiments.

Peptide	Area Unlabeled Peptide	Area Labeled Peptide	Ratio of Unlabeled/ Labeled Areas	Average Ratio
DLLLTSSYLSDSGSTG EHTK	55569	11347	4.3	4.4 ± 0.3
EDFDSLLQSAK	55125	10589	4.6	
IGDLQAFQGHGAGNL AGLK	266680	55646	4.2	
LDYLSSLK	232940	43462	4.7	
WCSWSLSQAR	45220	9689	4.1	

Table 6: Data used to calculate the abundance changes between the plasma membrane protein 4F2 cell surface antigen isolated from the unlabeled MXR MCF-7 cell line and the ^{13}C labeled drug susceptible MCF-7 cell line using the areas of the monoisotopic peaks from each peptide.

Accession #	Protein Name	Ratio MXR MCF-7 /MCF-7
tr|Q8IX16	ATP binding cassette protein ABCG2 (BCRP)	Not Detected in MCF-7 Cells
sp|P02786	Transferrin receptor protein 1	Not Detected in MCF-7 Cells
sp|Q01650	Large neutral amino acids transporter small subunit 1	7.9 ± 0.6
tr|Q9UIU0	Dihydropyridine receptor alpha 2 subunit	6.8 ± 0.4
sp|P08195	4F2 cell-surface antigen heavy chain	4.4 ± 0.3
sp|Q00610	Clathrin heavy chain 1	3.7 ± 0.4
sp|P04895	Guanine nucleotide-binding protein G(S), alpha subunit	3.5 ± 0.3
sp|P27105	Erythrocyte band 7 integral membrane protein	2.6 ± 0.3
sp|P54760	Ephrin type-B receptor 4	2.3 ± 0.3
sp|P17301	Integrin alpha-2	0.33 ± 0.03
tr|Q7Z3V1	Hypothetical protein DKFZp686D0452	0.3 ± 0.02
sp|P11166	Solute carrier family 2, facilitated glucose transporter, member 1	0.25 ± 0.02
tr|Q7Z7Q4	Tumor-associated calcium signal transducer 2	0.24 ± 0.08
sp|Q14344	Guanine nucleotide-binding protein, alpha-13 subunit	0.085 ± 0.01
sp|P26006	Integrin alpha-3	0.073 ± 0.006

Table 7: Protein abundance changes found when comparing the plasma membrane sub-proteomes of the unlabeled MXR MCF-7 breast cancer cell line and the 13C labeled drug susceptible MCF-7 parental cell line.

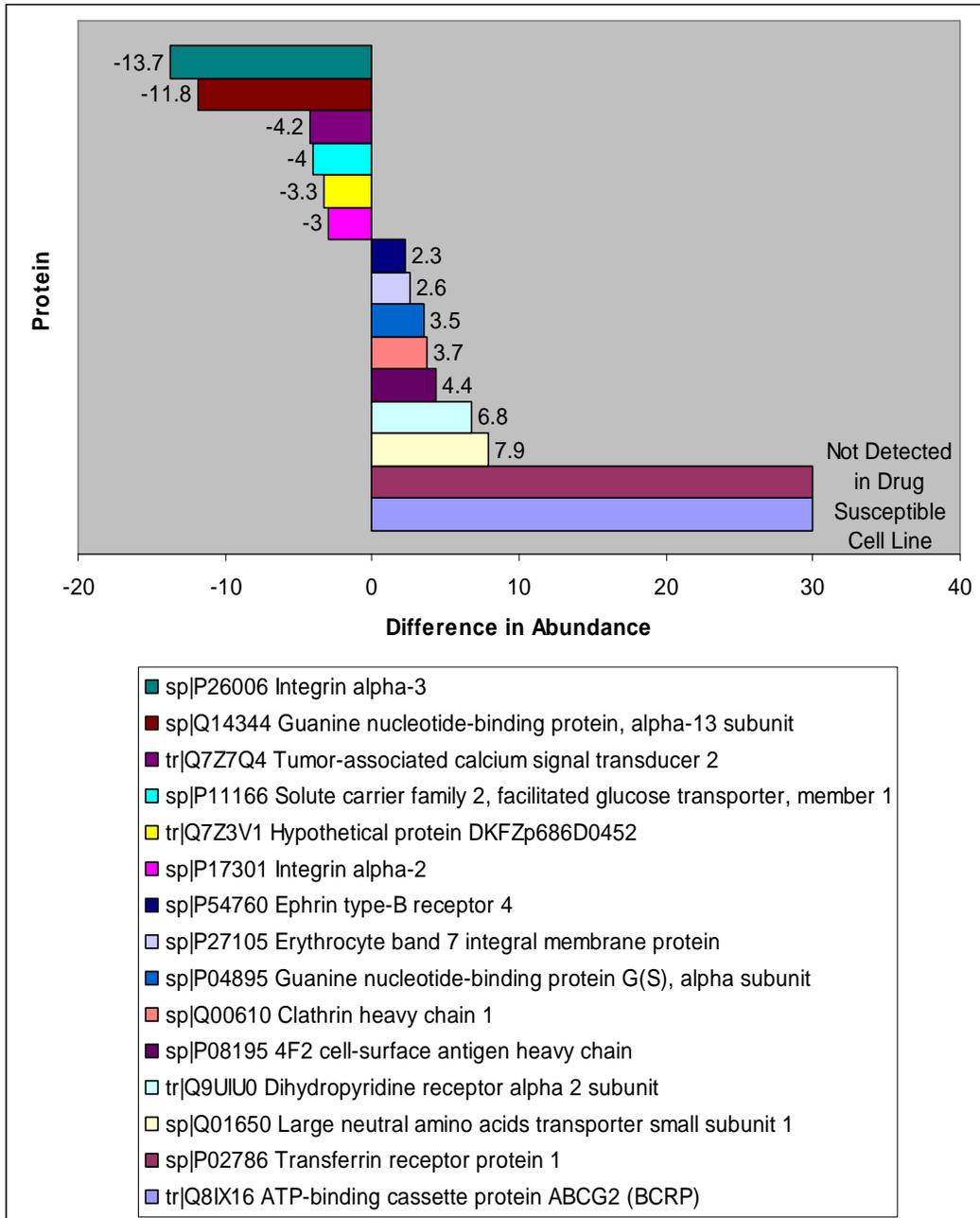**Figure 34:** Graph showing the relative changes in abundance of protein levels. The numbers represent relative fold increases in abundance levels with respect to the ratios of MXR MCF-7 protein abundance/ drug susceptible MCF-7 protein abundances. Positive values represent proteins whose abundance is higher in MXR MCF-7 cells and negative abundances represent proteins whose abundance is higher in the drug susceptible MCF-7 cell line.

with the integrin beta-1 subunit. The proteins summarized in **Table 7** can be classified into 4 different groups:

1. Cell Adhesion Proteins

    -   Integrin alpha-2

    -   Integrin alpha-2

    -   Hypothetical protein DKFZp686D0452 (beta integrin)

2. Transport Proteins

    -   ATP-binding cassette protein ABCG2 (BCRP)

    -   Solute carrier family 2, facilitated glucose transporter, member 1

    -   4F2 cell-surface antigen heavy chain

    -   Large neutral amino acids transporter small subunit 1

3. Proteins involved in Receptor Mediated Endocytosis

    -   Transferrin receptor protein 1

    -   Clathrin heavy chain 1

4. Signal Transduction Proteins and Receptors

    -   Guanine nucleotide-binding protein, alpha-13 subunit

    -   Guanine nucleotide-binding protein G(S), alpha subunit

    -   Erythrocyte band 7 integral membrane protein

- Ephrin type-B receptor 4

- Dihydropyridine receptor alpha 2 subunit

- Tumor-associated calcium signal transducer 2

The goal of this study was to identify the differences in protein abundances between the 2 cell lines and to use this data to elucidate the possible mechanisms of drug resistance for the MXR MCF-7 cell line. At first glance, the proteins listed in **Table 7** seem unrelated. The protein functions range from structural to the transport of materials and information.

The most well known contributor to drug resistance is the drug efflux protein P-glycoprotein (P-gp).[7] This protein is localized in the plasma membrane of cells and its mechanism involves ATP-dependant transport of materials out of the cell. This results in the reduction of intracellular accumulation of many types of anticancer drugs.[7; 8; 9] Although this is one of the most common causes of multidrug resistance, this protein was not detected in either cell line studied.

One of the major differences observed in the present study is the presence of the breast cancer resistance protein (BCRP), also called the ATP-binding cassette protein ABCG2. This protein is present in the MXR MCF-7 cell line but was not detected in the drug susceptible cell line. BCRP was first discovered in the

multidrug resistant breast cancer cell line MCF-7/AdrVp.[78] This cell line was selected by culturing MCF-7 cells in the presence of increasing amounts of adriamycin and verapamil.[89] Cells in which drug resistance is induced using doxorubicin have been found to overexpress the protein P-gp.[72; 73; 90; 91] Verapamil is an inhibitor of P-gp and by growing MCF-7 cells in the presence of both doxorubicin and verapamil, a different mechanism of resistance is achieved, which includes the overexpression of BCRP.

Like P-gp, BCRP is an ATP-driven efflux pump found in the plasma membrane. It has some substrates in common with P-gp and therefore can also facilitate multidrug resistance. Transfection of the drug susceptible MCF-7 cell line with an expression vector containing the full length BCRP cDNA clone resulted in cells which were resistant to mitoxantrone, daunorubicin, and doxorubicin, indicating that the overexpression of BCRP alone was enough to confer drug resistance.[78]

Like BCRP, transferrin receptor protein 1 was found in the MXR MCF-7 cell line but not detected in the drug susceptible cell line. Transferrin receptor protein 1 is responsible for the receptor mediated endocytosis of transferrin into the cell.[92; 93] Each transferrin protein binds 2 molecules of iron. Iron bound transferrin binds to transferrin receptor protein 1 and initiates uptake into the cell. This is the major mechanism for iron uptake by cells.[92]

Although an increase in transferrin receptor protein 1 or intracellular transferrin has not been associated with drug resistance, perhaps the increase in iron uptake by the cell is useful in an indirect way. Many proteins in the cell require iron in order to function properly. Proteins from the cytochrome P450 class of proteins contain a heme group and require iron for proper substrate binding. This class of proteins are classified as phase I detoxifying enzymes and have been previously shown to be a common mechanism of drug resistance.[7; 13; 14; 94] An increase in the intracellular expression of these enzymes would explain the benefit of increased uptake of iron. Clathrin abundance was increased by almost 4 fold in the MXR MCF-7 cell line. Clathrin is a protein used in the receptor mediated endocytosis process, and an increase in the abundance of this protein at the plasma membrane could be associated with an increase in the endocytosis process.

Guanine nucleotide-binding proteins, or G-proteins, are heterotrimeric proteins made up of 3 different subunits, called alpha, beta, and gamma. These proteins are located on the cytoplasmic face of the plasma membrane. The Guanine nucleotide-binding protein, alpha-13 subunit, was found to have almost a 12 fold decrease in abundance in the MXR MCF 7 cell line. This protein has not been previously associated with drug resistance, however it has been previously identified as an activator of apoptosis.[95; 96] Blocking of the apoptotic pathway is one way that drug resistance can be achieved, so this may be another mechanism used to achieve drug resistance by the MXR MCF-7 cell line.

The 4F2 cell-surface antigen heavy chain and large neutral amino acid transporter small subunit 1 proteins were both shown to be present in higher abundance in the MXR MCF-7 cell line. These proteins form heterodimers in the plasma membrane and are responsible for the uptake of amino acids into the cell. Since these proteins require both subunits for proper function, it makes sense that if there was an increase in the abundance of one of these proteins, the other would be increased also. Although the increase in these proteins has not yet been shown to confer drug resistance, it has been suggested that an increase in the expression of these 2 proteins would provide a selection advantage over other cells in the body because the cell would be able to survive in a nutrient restricted environment.[97]

Out of the six proteins whose abundances are lower in the MXR MCF-7 cell line, 3 of them are classified as integrins. Integrins are transmembrane spanning proteins which are responsible for the attachment of cells to extracellular matrix and cell-to-cell adhesion.[98; 99] They have a single transmembrane region and a large extracellular domain. They form heterodimers made up of alpha and beta subunits, both of which are required for proper function. Although there has been no data suggesting that a change in the abundance of integrins has any effect on drug resistance, there is data suggesting that changes in the expression of integrins can be responsible for metastasis and the spreading of tumor cells to other parts of the body through the circulatory system.[100; 101]

The erythrocyte band 7 integral membrane protein, also know as stomatin, showed an increase in abundance in the resistant cell line. Although the function of this protein is not known, it has been shown to associate with the glucose transporter solute carrier family 2, facilitated glucose transporter, member 1, also known as GLUT-1. [102; 103]These proteins co-immunoprecipitated with one another, suggesting that they physically interact in a complex.[102; 103] An increase in the expression of stomatin has also been associated with a decrease in the levels of glucose transport into the cell, without a decrease in the levels of GLUT-1 protein.[102; 103] In our experiments, we observed an increase in stomatin levels, but also a decrease in GLUT-1 levels. So here we have two different processes that potentially decrease intracellular levels of glucose. A decreased glucose level has not been associated with drug resistance, and is surprising in view of the energy needs of cancer cells.

Tumor-associated calcium signal transducer 2 is a cell surface receptor, which is widely expressed in human cancers.[104; 105] Its abundance was 4 fold lower in the MXR MCF-7 cell line, but its function and ligand are unknown. The dihydropyridine receptor alpha 2 subunit is a voltage gated calcium channel protein which had an increase in abundance in the MXR MCF-7 cell line. Both of these proteins appear to be involved in intracellular calcium signaling, but no link between these proteins has been reported and their function in relation to drug resistance is not immediately apparent.

The ephrin type-B receptor 4 and the guanine nucleotide-binding protein G(S), alpha subunit both have increased abundances in the MXR MCF-7 cell line. These proteins are both involved in signal transduction and other downstream signaling events. Unfortunately, without information on changes in protein concentrations in the interior of the cell, e.g., for the tumor-associated calcium signal transducer 2, and the dihydropyridine receptor alpha 2 subunit, the role of these membrane proteins is too general to determine their purpose in drug resistance.

Although the role played in drug resistance by the last four proteins discussed could not be surmised, functions of 11 of the 15 proteins of interest have been reasonably proposed. Nine of these have been reported by others to be related to drug resistance, metastasis, or increased resistance to harsh environments provided by the increase in the efficiency in nutrient uptake.

Of the 15 proteins exhibiting significant abundance changes, only BCRP has been previously reported as being associated with drug resistance.[106] It is suggested in this dissertation that by decreasing the abundance of guanine nucleotide-binding protein alpha-13 subunit, perhaps the tumor cell can increase its chances for survival while being treated with an anti-cancer agent by blocking one of the pathways leading to apoptosis and therefore achieving drug resistance. It is also suggested here that the purpose of increasing the abundance of transferrin receptor protein 1, and therefore increasing the intercellular concentration of iron, is to supply iron containing detoxifying enzymes, such as the cytochrome P450 family of proteins, with the iron

needed for proper function. The increase in detoxification enzymes has been linked with an increase in drug resistance.[7]

This dissertation suggests that at least one, and as many as three, mechanisms of drug resistance are achieved by the mitoxantrone drug resistant MCF-7 breast cancer cell line. As was stated earlier, the overexpression of BCRP alone is enough to confer drug resistance.[78] In the absence of further information on protein abundances within the cell, there is no way to determine whether guanine nucleotide-binding protein alpha-13 subunit, or transferrin receptor protein 1 are involved in drug resistance or some other process altogether. However, information about protein abundance differences can still be of use.

The overexpression of transferrin receptor protein 1 has been used for several different types of therapies. Researchers have been able to introduce reagents into cells by exploiting the transferrin receptor mediated endocytosis pathway. Transferrin binds many other metals besides iron and by complexing metals with therapeutic agents, these agents can be brought into the cell in this manner.[107] Anticancer agents have also been introduced into tumor cells by conjugating them to transferrin and the agents are therefore brought into the cell along with transferrin during Endocytosis.[107]

The overexpression of transferrin receptor protein 1 has also been used in gene therapy. Transferrin proteins can be synthesized containing long polylysine tails. These polylysine tails bind to plasmid DNA and this plasmid DNA isthen brought

into the cell during the endocytosis of transferrin. Using this technique, researchers

can introduce foreign genes into different systems.[108]

Conclusions and Prospectus

Mass spectrometry has established itself as an important analytical tool with many functions. It is used for the rapid detection and characterization of microorganisms and this use may someday be a vital tool in the defense against bio-terrorism and important for national security.[109] It is commonly used for the identification and analysis of proteins from complex biological systems.[41; 110] It can be used in combination with stable isotope labeling and differential densitometry techniques to determine relative protein abundance changes between separate samples.[73; 75] It can also be used (in combination with HPLC) in metabolic profiling experiments to determine the products formed from the breakdown of molecules through metabolic pathways.[111]

The methods improved upon and developed in this dissertation can be used to increase the understanding of the mechanisms and biological processes which occur in the plasma membrane. The plasma membrane is a major focal point for research in the area of drug design and targeted therapies, and although there is a significant amount of interest by biotechnology and pharmaceutical companies in the proteins found in the plasma membrane, relatively little is known about it due to the analytical challenges in isolation and separation.

Proteins identified can be used for identifying protein markers for early disease detection.[112] The information gained can also be used to design targeted therapies for the treatment of cancer and other diseases.[113] Information about protein expression in different disease states can help to elucidate the mechanisms of disease progression along with the causes of disease.

With the increases in the technology and advances in automation and bioinformatics, mass spectrometry may some day be used in doctors' offices for routine diagnostics and health examinations. Miniaturization of mass spectrometers and the decreases in instrument cost may allow routine screening of blood samples using the proteins from blood plasma or tissue biopsies taken from patients and this information could be critical for early diagnosis.

The improved method we developed for the isolation of plasma membranes in combination with mass spectrometry and proteomics allowed us to identify many proteins found in the plasma membrane.[48] The high level of plasma membrane enrichment achieved on the front end of the experiments allows for a much more detailed downstream analysis. This isolation method can almost be thought of as a first dimension of separation. Over 95% of the cellular components are separated, creating a fraction which is much less complex then the whole cell lysate. Combining this sub-cellular fractionation along with separation by SDS-PAGE and LC-MS provided a detailed analysis of the proteins from the plasma membrane.

Our experiments have shown that this technique works with both suspension cell culture and adherent cell cultures. The technique should also be transferable to tissue sample analysis and primary cell lines.[53; 54] Information obtained from this type of analysis may eventually be used to diagnose the presence and type of cancer in individual patients and can also be used to determine the mechanisms involved in drug resistance as is discussed in this dissertation.

The technique of *in vivo* metabolic labeling of cells in culture using stable isotopes allows proteins to be labeled prior to cell lysis.[72] Typically, proteins or peptides are labeled after cell lysis and the isolation of the desired protein fraction. By labeling during cell growth, as described in this dissertation, sample loss can be reduced due to the decrease in sample handling.

In closing, this dissertation outlines a method for the isolation, identification, and quantitation of plasma membrane proteins isolated from cell cultures. The information obtained from these types of comparative proteomic analyses can help to elucidate the mechanisms involved in drug resistance.

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