

USING PROTEOMICS AS A TOOL FOR
THE STUDY OF MICRONUTRIENT METABOLISM

by

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(Under the Direction of Arthur Grider, PhD)

ABSTRACT

Proteomics is the comprehensive and systematic study of the proteome, which is defined as the set of all expressed proteins in a cell, tissue or organism. The standard method for quantitative proteome analysis combines protein separation by two-dimensional gel electrophoresis (2DGE) with mass spectrometric (MS) identification of selected protein spots. Because these techniques allow one to study the full protein complement of a cell or tissue at the time of isolation, they are beneficial for identifying the pool of proteins whose expression is related to a particular treatment and to each other. This paper will present two proteomic studies of micronutrient metabolism. In the first study, the effect of zinc deficiency on protein expression in a colon adenocarcinoma (Caco-2) cell-culture model was investigated. In the second study, proteomics techniques were used to identify alterations in cellular protein expression, due to transfection with a selenoprotein construct (*pro-fs*) in Madin-Darby Canine Kidney cells.

INDEX WORDS: Two-dimensional gel electrophoresis, Zinc, Selenium, Caco-2, Madin-Darby Canine Kidney, Zinc-deficiency, *pro-fs*, HIV-1, proteomics, mass spectrometry

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DEDICATION

For my encouraging family and inspiring husband.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
CHAPTER	
1 INTRODUCTION	1
2 CHARACTERISTICS OF THE PROTEOME APPROACH	8
3 ZINC	17
4 ZINC DEFICIENCY AFFECTS PROTEIN EXPRESSION IN CACO-2 CELLS	38
5 SELENIUM	58
6 PROTEIN EXPRESSION OF MDCK CELLS TRANSFECTED WITH <i>PRO-FS</i> , A POTENTIAL SELENOPROTEIN MODULE	73
7 CONCLUSION.....	87
REFERENCES	91

CHAPTER 1

INTRODUCTION

The two minerals, zinc and selenium, are involved in a wide range of critical functions in the human body. Deficiencies in either of these micronutrients can result in numerous detrimental conditions, suggesting the importance of zinc and selenium as indispensable nutrients.

Two-dimensional gel electrophoresis (2DGE) in combination with mass spectrometry (MS) is a powerful method for the study of these micronutrients. This technique involves separating a complex mixture of proteins obtained from whole cells, tissues or organisms according to independent properties in two dimensions. The first dimension of 2DGE is isoelectric focusing (IEF), during which the proteins are separated based on their charge. In the second dimension, the proteins are separated orthogonally by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight (MW). The resulting patterns are oriented according to the Cartesian convention with the low, acidic isoelectric points to the left and the low molecular weights at the bottom. Once the separated proteins have been stained for visualization, selected protein spots are digested with trypsin and subjected to MS for identification. The proteins detected by this method will reflect the proteins expressed at the time of isolation (Graves and Haystead, 2002). Therefore, it is a powerful technique for studying the protein expression of cells under varying conditions.

Presented here will be two studies in which proteomic techniques were used to study micronutrient metabolism. In the first study, the effect of zinc deficiency on protein expression in

a colon adenocarcinoma (Caco-2) cell-culture model was investigated. In the second study, proteomics techniques were used to identify alterations in cellular protein expression, due to transfection with a selenoprotein construct (*pro-fs*), in Madin-Darby Canine Kidney cells.

1.1 Zinc

Zinc is a trace mineral present in all eukaryotic organisms where it plays important roles in growth and development, the immune response, neurological function, and reproduction. Zinc is present in more than 300 enzymes belonging to all six enzyme classes including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee and Falchuk, 1993).

The current Dietary Reference Intakes for zinc are listed for all age groups in Table 3.1. Zinc exhibits low toxicity. However, pharmacological doses of zinc antagonize copper absorption. The mechanism may be via induction of the intestinal metallothionein, which subsequently binds copper and limits its absorption. Reduced copper status, as measured by reduced erythrocyte superoxide dismutase activity, is considered the critical indicator for determining the tolerable upper intake level (UL) for zinc (Food and Nutrition Board, Institute of Medicine, 2002).

Zinc deficiency is a more frequent concern than that of toxicity. The need for zinc is greatest during periods of rapid growth, such as infancy, adolescence, and pregnancy, as well as during lactation. The pathological signs of zinc deficiency are growth failure, impaired parturition (dystocia), neuropathy, decreased and cyclic food intake, diarrhea, dermatitis, hair loss, bleeding tendency, hypotension, and hypothermia (Tapiero and Tew, 2003).

Exogenous zinc absorption occurs in the jejunum and ileum; where it is translocated into the enterocyte from the lumen, passes through the basolateral membrane, and is transported into

the portal circulation (Krebs, 2000). Two zinc transporters that have been identified which facilitate carrier-mediated zinc uptake into the intestinal cell, Zip4 and Zt1. Both of these transporter proteins are located at the apical surface of intestinal cells. The presence of Zip4 at the apical surface is responsive to dietary zinc, increasing with zinc deficiency and decreased during zinc sufficiency.

Although transcellular intestinal zinc transport is not fully understood, insight into the mechanism has been presented through recent studies. Cell culture transfection studies indicate that the cellular zinc status regulates the level of endocytosis for the Zip4 transporter. Under normal zinc conditions, Zip4 recycles rapidly between the plasma membrane and a perinuclear endosomal compartment. During zinc deficiency or supplementation recycling slows. Zip4 shifts to the plasma membrane during deficiency and shifts to the intracellular endosomal vesicles during supplementation. The presence of the transporter at the plasma membrane corresponds with changes in zinc transport (Kim et al., 2004; Wang et al., 2004).

Transfection studies using cell culture identified Zt1 as another zinc transport protein potentially involved in intestinal zinc uptake (Cragg et al., 2002). This transporter was also regulated by zinc. However, its upregulation occurs with zinc supplementation.

The intracellular transport of zinc from the apical to basolateral intracellular surface for transport to the portal circulation is not known at this time. The extrusion of zinc from the cell into the portal circulation may occur through another zinc transporter protein, Znt-1 (Palmiter and Findley, 1995; McMahon and Cousins, 1997).

The mechanism of zinc uptake by systemic cells supports the presence of two major pathways, (1) a saturable receptor-mediated co-transport of zinc-albumin by transcytotic vesicles and (2) a non-saturable co-transport with ligands (mainly albumin and histidine) through

intercellular junctions (Tibaduiza and Bobilya, 1996). Zinc may also enter complexed with cysteine or histidine via a sodium/amino acid co-transport mechanism (Gachot, 1991). Despite these general conclusions, the mechanisms of the different transport and binding steps are, for most cell types, only partially solved (Reyes, 1996).

Because it is difficult to control various parameters in the intestinal system of the whole animal, *in vitro* systems have been developed to define specific mechanisms of zinc uptake and transport. Human colonic adenocarcinoma cells (Caco-2 cells) are valuable *in vitro* tools for studies related to intestinal cell function and differentiation. Caco-2 cells are able to differentiate into small intestine-like enterocytes; they polarize when plated on Transwells® membrane supports, allowing for the study of apical to basolateral and basolateral to apical zinc transport.

We investigated the effect of zinc deficiency on protein expression in the colon adenocarcinoma (Caco-2) cell-culture model. The aims of this investigation included, 1) the identification of expressed proteins of colon adenocarcinoma cells in response to incubation in zinc deficient medium, compared to control; and 2) to gauge the effectiveness of proteomics techniques (2DGE and MS) for investigating zinc metabolism. The effect of dietary zinc depletion on the protein profile of the small intestine could provide informative clues to understanding the role for zinc in the cell cycle regulation, as well as proteins involved in the uptake of zinc.

1.2 Selenium

Selenium (Se) is an essential micronutrient in the diet of humans and other mammals. It is quickly becoming recognized as one of the more promising cancer chemopreventive agents. There are strong indications that it has a role in reducing viral expression (Beck et al., 2003), in preventing heart disease and other cardiovascular and muscle disorders (Neve, 1996), and in

delaying the progression of the acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-infected patients (Baum et al., 1997). Additional evidence suggests that selenium may have a role in mammalian development, in immune function, in male reproduction, and in slowing the aging process (Rayman, 2000).

Physiologically, Se functions in the form of selenium-dependent enzymes, incorporating selenium as selenocysteine in the catalytic centers of the proteins. Nearly three dozen selenoproteins have been identified (Rayman, 2000). Selenocysteine, a seleno-analog of cysteine, is cotranslationally incorporated into selenoproteins at UGA codons, but only when specific stem-loop structures are present in the 3' untranslated region of the mRNAs. Conserved primary sequence and secondary structural features in these elements are required for selenocysteine incorporation. When these structures, termed Sec insertion sequence (SECIS) elements, are absent or disrupted, the default function of UGA codons, termination of protein synthesis, is utilized (Copeland, 2003).

Selenium is found in organic (selenocysteine and selenomethionine) and inorganic (selenite and selenate) forms. The organic form is found predominantly in grains, fish, meat, poultry, eggs and dairy products, and enters the food chain via plant consumption. Some nuts, in particular Brazil nuts and walnuts, are also very good sources of selenium. Animals that eat grains or plants that were grown in selenium-rich soil have higher levels of selenium in their muscle.

The current Dietary Reference Intakes for selenium are listed for all age groups in Table 4.1 (Food and Nutrition Board, Institute of Medicine, 2000). The Food and Nutrition Board set the tolerable upper level (UL) for selenium at 400 $\mu\text{g}/\text{day}$ in adults based on the prevention of hair and nail brittleness and loss and early signs of chronic selenium toxicity. At doses above

900µg per day, selenium produces a toxic syndrome consisting of dermatitis, loose hair, diseased nails, and peripheral neuropathy (Yang et al., 1983). The most recent RDA is based on the amount of dietary selenium required to maximize the activity of the antioxidant enzyme glutathione peroxidase in blood plasma (Food and Nutrition Board, Institute of Medicine 2000).

Selenium deficiency appears to enhance the virulence or progression of some viral infections (Beck, 1999). The increased oxidative stress resulting from selenium deficiency may induce mutations or changes in the expression of some viral genes. The significance of the role of Se in disease progression has been documented in viruses including hepatitis b, coxsackievirus/Keshan disease, viral hemorrhagic fever, and the mouse mammary tumor virus. In these cases, Se deficiency combined with a viral cofactor causes the disease to progress (Reviewed by Taylor et al., 1997).

There appears to be a unique interaction between selenium and the virus that causes AIDS. A progressive decline in plasma or serum selenium levels, paralleling the loss of CD4+T cells, has been widely documented in HIV-1 infections (Look et al., 1997). Selenium is a potent inhibitor of HIV replication in vitro (Sappey, 1994). The mechanisms by which selenium influences the progression of these diseases remains unknown, but the potential significance of the role of selenium in AIDS progression may be similar to that of the other viruses.

Based on an analysis of the genomic structure of HIV-1, Taylor et al. (1994) demonstrated that several regions overlapping known HIV genes have the potential to encode selenoproteins. It is hypothesized that during HIV infection, several viral selenoproteins are expressed by ribosomal frameshifting and/or suppression of termination codons, and synthesis of these viral selenoproteins deplete the selenium pool of the host. One predicted selenium-incorporating site found by Taylor et al. (1994), later termed *pro-fs* (due to expression by a –1

frameshift from the protease coding region), has been found to be a potent activator of HIV-1 replication in transfected Madin-Darby Canine Kidney (MDCK) cells (Taylor et al., unpublished).

The Madin-Darby Canine Kidney (MDCK) cell line is an established line of canine kidney cells that has been used in several laboratories to study virus-host cell relationships. When grown in tissue culture, MDCK cells have the morphological properties of distal tubular epithelial cells (Rindler, 1979).

Two-dimensional gel electrophoresis (2DGE) was used to identify alterations in cellular protein expression, due to transfection with the pro-fs construct, in Madin-Darby Canine Kidney (MDCK) cells. Elucidation of the importance of novel viral selenoproteins may improve our understanding of HIV.

CHAPTER 2

CHARACTERISTICS OF THE PROTEOME APPROACH

Proteomics, first coined in 1995, is the comprehensive and systematic study of the proteome, which is defined as the set of all expressed proteins in a cell, tissue or organism. The emerging field of proteomics has grown out of the combination of two-dimensional gel electrophoresis (2DGE) for protein separation and quantification, and mass spectrometry (MS) for the identification of separated proteins.

2.1 Proteomics Origins

The first protein studies that can be called proteomics began nearly thirty years ago with the introduction of the two-dimensional gel by O'Farrell (1975), Klose (1975), and Scheele (1975), of who began mapping proteins from *Escherichia coli*, mouse, and guinea pig, respectively. Although many proteins could be separated and visualized, they could not be identified. With the development of mass spectrometry (MS) technology, however, it is now possible to sequence thousands of peptides representing hundreds to thousands of proteins from a single sample taken directly from a cell lysate (Anderson and Mann, 2000).

Together, 2DGE and MS now represent an integrated technology that is considered the gold standard for proteome analysis. Using 2DGE, several thousand protein species can be separated, detected and quantified in a single operation. Thanks to MS, hundreds of the detected proteins can be identified in a highly automated fashion by sequential analysis of the peptide mixtures generated by digestion of individual gel spots. Furthermore, the additional information

obtained from a 2D gel (e.g. isoelectric focusing point and molecular mass) adds validity to MS based protein identification.

2.2 Genome Information

The growth of proteomics is a direct result of advances made in large-scale nucleotide sequencing of expressed sequence tags and genomic DNA. Without this information, identification of proteins would not be possible, regardless of advances in MS. Protein identification relies on the presence of some form of database for the given organism (Shevchenko et al., 1996). In 1995, the first complete genome of an organism was sequenced, that of *Haemophilus influenzae* (Fleischmann et al., 1995). To date, hundreds of genomes have been completed, including the human genome (Venter et al., 2001; Collins et al., 2001).

2.3 Types of Proteomics

Proteomics can be classified into three types: functional proteomics, structural proteomics, and protein expression proteomics (Reviewed by Lau et al., 2003).

2.3.1 Functional Proteomics

Analyzing protein profiles at subcellular sites is an important approach in understanding the functional organization of cells at the molecular level. In this respect, information about the specific subcellular localization of a protein may help to elucidate its function. The combination of protein identification by mass spectrometry with fractionation techniques such as immunoprecipitation or chromatography for the enrichment of particular subcellular has been termed 'subcellular proteomics' (Dreger, 2003). The analysis of proteins at the subcellular level is the basis for monitoring important aspects of dynamic changes in the proteome such as protein translocation.

2.3.2 Structural Proteomics

The main goal of structural proteomics is to map out the structure of protein complexes or the proteins present in a subcellular localization or an organelle (Blackstock and Weir, 1999). This approach can identify all the proteins within a compartment such as mitochondria, chloroplast and nuclei, or protein-protein interactions in a complex such as the transcriptome, where many proteins work as a gigantic complex during transcription.

2.3.3 Protein Expression Studies

Expression proteomics is the quantitative study of protein expressions between samples. In this approach, protein expressions of the entire proteome or of subset proteomes can be compared. Novel proteins (e.g. disease-specific biomarkers) can also be identified.

Protein expression of different samples, i.e. control and treatment, can be qualitatively and quantitatively compared. The appearance or disappearance of specific protein spots indicates differential protein expression, while the degree of spot intensity provides quantitative information about protein expression levels.

2.4 Technology and Methodology

A typical proteomics experiment can be broken down into the following steps: (i) solubilization of proteins from the sample; (ii) separation of the proteins by 2DGE; (iii) digitization of 2D gels and computer-assisted analysis of protein spot patterns; (iv) determination of specific attributes of the proteins of interest by MS; and (v) identification of those proteins by database inquiry (Giorgianni et al., 2003, Lau et al., 2003).

2.4.1 Sample Preparation

Sample preparation prior to 2-DGE is critical for producing meaningful, reproducible results. The principal to follow is to avoid causing any chemical modification of proteins, since

any change in charge will be detected on the gel and will result in an aberrant pattern. No single method of sample preparation can be universally applied due to the diverse nature of samples which are analyzed by 2DGE.

Cells grown *in vitro* in suspension culture can be harvested by centrifugation, washed in phosphate-buffered saline (PBS), and solubilized. The ideal solubilization procedure for 2DGE would result in the disruption of all non-covalently bound protein complexes and aggregates into a solution of individual polypeptides (Dunn, 1993). The primary separation of proteins employed high concentrations of the chaotropic agents, urea and thiourea, to increase effectiveness of solubilization.

2.4.2 Isoelectric Focusing (IEF)

IEF is an electrophoretic method that separates proteins according to their isoelectric points (pI). As amphoteric molecules, proteins can carry a positive, negative, or zero net charge depending on the pH of their surroundings. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. This net charge is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl- termini.

The isoelectric point is the specific pH at which the net charge of the protein is zero. In the presence of a pH gradient and electric field, proteins possessing a net positive or negative charge will migrate toward the appropriate electrode until they reach a position in the pH gradient at which the pH equals the pI. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is what is referred to as the focusing effect of IEF.

The degree of resolution is determined by the slope of the pH gradient and the electric field strength. IEF is therefore performed at voltages typically in excess of 1,000 V. When the

proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current, usually below 1 mA. IEF of a given sample in a given electrophoresis system is generally performed for a constant number of volt-hours, which is the product of the voltage and the hours elapsed at that voltage.

2-DGE was created by carrier ampholytes. However, the resulting patterns were insufficiently reproducible since electrophoresis is performed under denaturing conditions, which prolongs the running time and destabilizes the pH gradient. Gradient drifting with prolonged isoelectric focusing time causes the loss of almost all basic and some of the acidic proteins.

Immobilized pH gradient (IPG) gels, introduced by Bjellqvist et al. (1982), are created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast. The immobilized strips are now commercially available in various lengths (7 cm, 13 cm, 18 cm, and 24 cm), and cover various pH ranges, including broad range pH 3.10, narrow ranges pH 4.7 and pH 6.11, and a range of a single pH increment. The IPG strips have a higher loading capacity for protein, compared to tube gels with carrier ampholyte-generated pH gradients. The main protocol for the use of IPG for 2DGE was published by Görg et al. (1988).

IEF is performed with the IPG strips placed horizontally on a flatbed electrophoresis unit. Isoelectric focusing requires efficient cooling for close temperature control, which can be effectively achieved on a horizontal ceramic cooling plate connected to a thermostatic circulator or a Peltier cooling plate. IEF requires high field strengths to obtain sharply focused bands, thus high voltages must be applied. A flatbed design is the most economical way to meet the necessary safety standards required to operate at such high voltages.

After IEF the IPG strips are equilibrated with 6 M urea, 30% glycerol, 2%, 1.55 M Tris, 2 mM tributylphosphine, for 25 min at room temperature; and applied onto vertical sodium dodecyl sulphate (SDS)-polyacrylamide gels. The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation.

2.4.3 Second-Dimension SDS PAGE

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) is an electrophoretic method for separating polypeptides according to their molecular weights (MW). There is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide micelle, determined by the polyacrylamide percentage (Reviewed by Graves and Haystead, 2002). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 grams SDS per gram protein. By binding to the protein, a complex is formed in which the charge-to-mass ratio is constant. The SDS also disrupts hydrogen bonds, blocks hydrophobic interactions, and partially unfolds the protein molecules, minimizing differences in molecular form by eliminating the tertiary and secondary structures. The proteins are totally unfolded when a reducing agent such as DTT is employed. The disulphide bonds, which can form between cysteine residues, are cleaved, and the polypeptides become flexible rods of negative charges with equal "charge densities," or charge per unit length.

The ability to generate reproducible 2-D patterns is as important as the absolute protein resolving capacity. It is, therefore, essential to perform all of the steps involved in 2DGE in as careful and controlled a manner as possible to maximize reproducibility (Dunn, 1993).

2.4.4 Protein Visualization

Once electrophoresis is complete, the gel must be analyzed to obtain information on the position and quantity of each protein. Because most proteins are not directly visible, the gel must be processed to determine the location and amount of the separated proteins. The most common analytical procedure is staining. All standard procedures of protein staining are used in proteomic studies. However, some of them are not applicable for mass-spectrometry methods of protein identification due to irreversible modification of amino acids. Photochemical methods of staining with silver (Nesterenko et al., 1994; Shevchenko et al., 1996), colloid Coomassie solution (Neuhoff et al., 1988), and the fluorescent dye Sypro Ruby (Yan et al., 2000) are the most popular and widely used methods of staining. Once stained, the gel image is digitized for analysis by a variety of image analysis programs.

Coomassie Blue staining is based on the binding of the dye Coomassie Brilliant Blue R250, which binds nonspecifically to virtually all proteins. Although Coomassie Blue staining is less sensitive than silver staining, it is widely used due to its convenience. Coomassie Blue binds to proteins approximately stoichiometrically, so this staining method is preferable when relative amounts of protein need to be determined by densitometry. For most SDS and native gels, separated proteins can be simultaneously fixed and stained in the same solution, by soaking the gel in a solution of the dye. Any dye that is not bound to protein diffuses out of the gel during destaining steps, removing the background prior to drying and documenting. The proteins are detected as blue bands on a clear background.

Silver Staining is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels (Amersham Biosciences, 2004). This sensitivity, however, comes at the expense of high susceptibility to interference from a number of factors. Precise timing, high-

quality reagents, and cleanliness are essential for reproducible, high-quality results. In silver staining, the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by protein.

Sypro Ruby protein gel stain has two excitation peaks at ~280 nm and ~450 nm, and has an emission maximum near 610 nm. Stained proteins can be visualized using a variety of excitation sources including a 300 nm UV or blue-light transilluminator, or laser-based systems. Sypro Ruby protein gel stain also has exceptional photostability and a long emission lifetime, allowing for long exposure times while minimizing background fluorescence (Rev, 2004).

2.4.5 Image Analysis and Documentation

After 2DGE and protein visualization by staining, images of gels are digitized for computer analysis by an image scanner or fluorescent scanner, and are then subjected to analysis by special image analysis software Phoretix 2D Advanced software (v6.01). The 2-D patterns are very complex, and special software tools may be required to find differentially expressed proteins in a series of gels, such as up and down-regulated proteins, post-translational modified proteins. Image spots on the gels are initially detected, manually edited and then matched. The reliability of quantitative determinations of protein amounts in spots is largely dependent on the protein detection technique applied. Usually, only significantly up/down-regulated spots or appearing/disappearing spots are selected for analysis with mass spectrometry.

2.4.6 Mass Spectrometry (MS) and Protein Identification

Today MS is overwhelmingly used as the technology base for protein identification from 2D gels due to its rapid and sensitive protein identification and quantification. MS enables protein structural information, such as peptide masses or amino acid sequences, to be obtained.

This information can be used to identify the protein by searching nucleotide and protein databases. In the last decade, the sensitivity of analysis and accuracy of results for protein identification by MS have increased by several orders of magnitude (Anderson and Mann, 2000; Pandey and Mann, 2000). It is now possible to identify thousands of proteins from microgram quantities in a single day and quantify relative protein abundances.

There are three different types of MS data that can be used for database search. They are (1) molecular weights of peptides that can be used for Peptide Mass Mapping, (2) combination of mass data and partial amino acid sequence that can be used for Sequence Tag, and (3) tandem mass spectrometry data that are used for MS/MS fragmentation ion search (British Mass Spectrometry Society, 2003).

CHAPTER 3

ZINC

3.1 Importance of zinc as a nutrient

Human zinc deficiency was first recognized by Dr. Ananda Prasad, while studying young Egyptian men exhibiting anemia, dwarfism, hypogonadism, dry skin, and mental lethargy (Prasad et al., 1961). Supplementation with iron ameliorated the clinical symptoms, but could not fully account for the retarded growth or gonadal failure, that occurred in zinc-deprived animals. Subsequent treatment trials on patients in Egypt established that zinc deficiency was the primary cause of these symptoms (Prasad et al., 1963). Two major findings from these early studies indicate the importance of zinc in human health. First, zinc concentrations in the plasma, hair, erythrocytes, urine and stool were decreased whereas the ^{65}Zn turnover rate was increased in these growth-retarded patients compared to healthy subjects. Secondly, patients treated with supplemental zinc exhibited a growth rate faster than those who received iron supplementation or an animal protein diet (Prasad, 2003). Numerous studies have since characterized the role zinc plays in cellular metabolism.

Zinc is present in more than 300 enzymes belonging to all six enzyme classes including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee and Falchuk, 1993). Among these enzymes, zinc plays catalytic, co-catalytic or structural roles (Vallee and Falchuk, 1993; Reviewed by McCall et al., 2000). In a catalytic zinc site, the zinc ion directly

participates in the bond-making or -breaking step, as in carbonic anhydrase (Liljas et al., 1972). The additional zinc site is co-catalytic, as in alkaline phosphatases, when more than one zinc-containing subunit constitutes the catalytic structure (Vallee and Falchuk, 1993; Butterworth, 1983). In a cocatalytic zinc site, there are several metal ions bound in proximity to one another, where one plays a catalytic role and the other metal ions enhance the catalytic activity of the site (Vallee and Auld, 1993). Finally, in structural zinc sites, the zinc ion mainly stabilizes the tertiary structure of enzymes by forming a tetrahedral coordination with cysteine or histidine ligands. In all cases, removal of the bound zinc can lead to a loss of enzymatic activity.

In addition, zinc plays a key role in gene expression. Virtually all DNA-binding proteins are zinc finger proteins encoded by approximately 1% of the human genome (Choo and Klug, 1997). Zinc finger proteins share a common DNA recognition helix motif, which is formed by zinc binding to histidine and/or cysteine on the peptide. These transcription factors alter the rate of transcription and the cellular abundance of certain mRNAs. Ionic zinc also regulates metallothionein gene transcription via interaction with metal response elements (MRE) in the promoter (Bi et al., 2004). Since zinc is present in numerous cellular pathways, many physiological systems may be affected by zinc status.

Zinc may function as a component of the mechanism for controlling intracellular vesicular compartment translocation. The recycling of several of the zinc transporters, between the plasma membrane and intracellular vesicular compartments is zinc dependent (Lonnerdal, 1989). Also, several of the proteins involved in the translocation machinery contain zinc-finger motifs. These proteins include protein kinase C and several of the tyrosine kinases (Mathews and Sunde, 2002).

3.2.1 Biomarkers of zinc intake and status

Biomarkers of zinc intake and status are inadequate. This is due to 1) the small decrements in tissue zinc that are associated with zinc-deficient morbidity and impaired development; 2) the effectiveness of homeostatic mechanisms in maintaining tissue, notably, from a biomarker perspective, circulating plasma/serum zinc concentrations; and 3) the extraordinarily widespread dependence on zinc of so many aspects of biology including its involvement in gene expression and a variety of aspects of cellular growth and replication (Hambridge, 2003). Elucidation of the prevalence and the clinical effects of milder zinc deficiency states have depended to a very large extent on the results of well-designed and executed, controlled, randomized intervention studies with dietary zinc supplements (Hambridge, 2003). The measurement of fractional zinc absorption is currently used for determining the level of dietary zinc necessary for normal zinc status (King, 2001). The data from this technique was used to determine the dietary reference intake values for zinc (Food and Nutrition Board, Institute of Medicine, 2002).

3.1.2 Pathological consequences of zinc deficiency

Due to the ubiquity of this metal in biology, the detectable features and functional abnormalities of zinc deficiency are diverse. Zinc deficiency states are associated with anorexia and alterations of the epidermal, gastrointestinal, central nervous, immune, skeletal and reproductive systems (Tapiero and Tew, 2003).

Severe zinc deficiency in humans causes prominent skin lesions around the body orifices and extremities. In zinc-deficient rats, non-scarring alopecia, diminished subcutaneous tissue, and nude skin have been documented (Hambridge, 2000). Parakeratosis and skin fragility, are found in pigs, calves and lambs (Apgar and Fitzgerald, 1985).

Diarrhea is another clinical feature of zinc deficiency. Possible causes of this complication include immune depression, and reduced water/electrolyte permeability in intestinal mucosal cell membranes (Ghishan, 1984). The loss of zinc from the intestine increases during the diarrhea state, worsening the disease, and may be lethal if untreated. Zinc supplementation to children in developing countries has been shown to significantly reduce the severity and frequency of diarrhea (Bhutta et al., 1999).

The central nervous system is usually affected by zinc deficiency in the stages of early development. Effects include deficits in motor skill, cognition and emotionality. Evidence of improved brain development attributable to improved zinc status has been derived from activity levels in young children in India (Sazawal et al., 1996) and Guatemala (Bentley et al., 1997).

In zinc deficient animals, all major branches of the immune system are compromised (Mills, 1989). Suboptimal intake caused marked atrophy of the thymus (Ripa and Ripa, 1995); reduced T-lymphocytes, phagocytes, and leukocytes (Prasad 1998); depressed antibody-mediated, cell-mediated, and delayed hypersensitivity responses (Fraker, 2000); and decreased natural killer cell activity (Shankar and Prasad, 1998). These effects can be alleviated with zinc supplementation.

Zinc deficiency also adversely affects reproduction in both males and females. Zinc status also affects fetal growth and pregnancy outcome. Low plasma zinc in mothers is associated with delivery complications and retarded fetal growth. Overt zinc deficiency is associated with teratogenesis (Hambridge, 2000). Zinc deficient males exhibit hypogonadism and delayed sexual maturation (Prasad et al., 1961).

3.1.3 Zinc deficiency induced by malnourishment

Although zinc deficiency can affect virtually every organ system in the body, tissues which have a requirement for rapid cell division are the most susceptible to zinc deficiency (Mills, 1989). Consequently, the most severe effects on zinc deprivation are found during periods of rapid growth, such as infancy, adolescence, and pregnancy, as well as during lactation. Individuals with malabsorptive syndromes including sprue, Crohn's disease, and short bowel syndrome are at risk for malabsorption of zinc and increased urinary zinc losses (Food and Nutrition Board, Institute of Medicine, 2002). Individuals whose diet contains high concentrations of phytate, a powerful chelator that reduces the bioavailability of metallic elements, are at risk of zinc deficiency (Reviewed by Tapiero and Tew, 2003). Severe clinical symptoms have developed from parenteral nutrition without supplemented zinc, in some premature infants, and in patients with protein energy malnutrition (Mills, 1989). For a more complete listing of factors predisposing to zinc deficiency, see figure 3.1.

3.1.4 Genetic disorder-related zinc deficiency

The human genetic disorder acrodermatitis enteropathica (AE) is a long recognized disease of zinc metabolism. Clinical symptoms of this disorder are very similar to severe dietary zinc deficiency including acute dermatitis, alopecia, respiratory tract infection, diarrhea, and arrested growth (Van Wouwe, 1989). Manifestation of this disorder usually occurs during early infancy. This autosomal recessive disorder causes classic symptoms of zinc deficiency that are ameliorated by dietary zinc supplementation that must be continued throughout the lifetime (Baudon, 1978; Krieger, 1982). This success of treatment with zinc supplementation is consistent with the finding of reduced, but not eliminated, uptake of ^{65}Zn by the intestine from AE patients (Lombeck, 1975; Atherton, 1975), and the reduced uptake and total content of zinc in AE

fibroblasts (Grider, 1998). Recent genetic mapping localized the AE gene to chromosome 8q24.3 (Wang, 2001) and led to its identification as a member of the ZIP superfamily (Wang, 2002; Kury, 2002). This gene, named *hZIP4* (the Human Genome Organization Nomenclature Committee named this gene *SLC39A4*), was found to be expressed in enterocytes and to reside in the plasma membrane. Mutations in *hZIP4* were detected in AE patients (Wang, 2002; Kury, 2002). Dufner-Beattie et al. (2003) examined the function and expression of mouse *ZIP4*. They demonstrated that *mZIP4* encodes a zinc transporter, that this gene is expressed in intestine and embryonic yolk sac, that *ZIP4* localizes to the apical surface of enterocytes and visceral endoderm cells, and that the expression of this gene and its protein product is dynamically regulated by zinc. The results of these studies support the hypothesis that *ZIP4* is a zinc transporter that plays an important role in zinc homeostasis, a process that is defective in acrodermatitis enteropathica in humans.

3.1.5 Pathological consequence of excessive zinc

Zinc exhibits low toxicity. Manifestations of overt toxicity are due to the use of supplements, occurring with long-term exposure to 100-300 mg of zinc per day (6-20 times the RDA). Extremely high zinc intake will cause vomiting, epigastric pain, lethargy, and fatigue (Food and Nutrition Board, Institute of Medicine, 2001). Metal fume fever has been reported after the inhalation of zinc oxide fumes. Profuse sweating, weakness, and rapid breathing may develop within 8 hours of zinc oxide inhalation and persist 12-24 hours after exposure is terminated (Food and Nutrition Board, Institute of Medicine, 2001). Intranasal zinc is known to cause a loss of the sense of smell (anosmia) in laboratory animals (McBride, 2003), and there have been several case reports of individuals who developed anosmia after using intranasal zinc gluconate (Jafek et al., 2003; DeCook and Hirsch, 2000).

Reduced copper status, as measured by reduced erythrocyte superoxide dismutase activity, is considered the critical indicator for determining the tolerable upper intake level (UL) for zinc (Food and Nutrition Board, Institute of Medicine, 2002). Evidence of the interaction between zinc and copper derives from the therapeutic effect of zinc in reducing copper absorption in patients with Wilson's disease (Sandstead, 1989). This action includes the induction of intestinal metallothionein by zinc and the subsequent binding of excess copper by this metalloprotein, which may limit transcellular copper absorption.

3.2 Metabolic Aspects of Zinc

Since zinc is so important to animal welfare, the dietary sources of zinc, metabolic pathways that use zinc, and the dynamic distribution of zinc in the body have been well studied. The adult human body contains about 1.5 to 2.5 grams of zinc, with over 95% bound to proteins within cells and cell membranes (Mills, 1989). Unlike other trace elements such as iron, zinc does not accumulate in the body to form permanent stores. Studies in experimental animals and humans have shown that the whole-body content of zinc remains relatively constant over a wide range of intakes. Adjustments in gastrointestinal zinc absorption and endogenous excretion appear to be the primary means of maintaining zinc homeostasis (Krebs, 2000). The adjustments in gastrointestinal zinc absorption and endogenous excretion are synergistic. Shifts in endogenous excretion appear to occur quickly with changes in intake just above or below optimal intake. The absorption of zinc responds more slowly, but it has the capacity to cope with large fluctuations in intake. With extremely low zinc intakes or with prolonged marginal intakes, secondary homeostatic adjustments may augment the gastrointestinal changes. These secondary adjustments include changes in urinary zinc excretion, a shift in plasma zinc turnover rates and,

possibly, an avid retention of zinc released from selected tissues, such as bone, in other tissues to maintain function (Krebs, 2000).

3.2.1 Sources and Bioavailability of Zinc

The dietary sources of zinc vary widely. Zinc is abundant in red meats, certain seafood, and whole grains. Because zinc is mainly located in the germ and bran portions of grains, as much as 80 percent of the total zinc is lost during milling. Many breakfast cereals are fortified with zinc (Food and Nutrition Board, Institute of Medicine, 2002). The maximal fractional absorption of zinc in humans is 40% from an aqueous solution, with absorption in the presence of food ranging from 1% to 40%.

The recommended daily allowance (RDA) of zinc for different age groups is listed in Table 3.1 (Food and Nutrition Board, Institute of Medicine, 2002). Adequate intake and efficient absorption of dietary zinc are critical for a healthy zinc status, as discussed in section 3.1.3. Several dietary factors affect zinc absorption. Many ligands, especially phytates and fibers, reduce the bioavailability of zinc (Food and Nutrition Board, Institute of Medicine, 2002). In a 63-day study involving men in a metabolic ward, phytate consumed in liquid formula diets depressed zinc absorption (Turnlund et al., 1984). Prenatal iron supplements (38-65 mg/day of elemental iron) may adversely influence zinc absorption during pregnancy. This has led to the recommendation of zinc supplementation for pregnant and lactating women taking more than 60 mg/day of elemental iron (Fung, 1997; O'Brien, 2000). Increasing the calcium intake of postmenopausal women by 890 mg/day in the form of milk or calcium phosphate (total calcium intake 1,360 mg/day) reduced zinc absorption and zinc balance in postmenopausal women (Wood and Zheng, 1997), but increasing the calcium intake of adolescent girls by 1,000 mg/day in the form of calcium citrate malate (total calcium intake 1,667 mg/day) did not affect zinc

absorption or balance (McKenna et al., 1997). Interactions with copper are discussed in section 3.1.5.

3.2.2 Zinc absorption, transport, tissue distribution and excretion

Exogenous zinc absorption occurs in the jejunum and ileum; where it is translocated into the enterocyte from the lumen, passes through the basolateral membrane, and is transported into the portal circulation (Krebs, 2000). It is generally assumed that an intraluminal transition occurs to allow hydrophilic zinc to be transported across enterocytes as free ions (Cousins, 2000). Carrier mediated diffusion occurs in a time, pH, and temperature dependent manner.

Intestinal zinc transport has been characterized (Reyes, 1996). A review of literature indicates that, in all cells, zinc interacts with extracellular binding sites, which are likely to include binding sites involved in the subsequent translocation of this ion into the cell interior. Inside the cell, zinc binds to cytosolic and organelle binding sites or is taken up by intracellular organelles. Candidate zinc transporters involved in transcellular transport are discussed in Section 3.3.

The mechanism of zinc uptake by cells supports the presence of two major pathways, (1) a saturable receptor-mediated co-transport of zinc-albumin by transcytotic vesicles and (2) a non-saturable co-transport with ligands (mainly albumin and histidine) through intercellular junctions (Tibaduiza and Bobilya, 1996). Zinc may also enter complexed with cysteine or histidine via a sodium/amino acid co-transport mechanism (Gachot, 1991). Despite these general conclusions, the mechanisms of the different transport and binding steps are, for most cell types, only partially solved (Reyes, 1996).

Plasma is the major exchange pool for systemic zinc requirements. The zinc concentration in plasma is about 15 $\mu\text{mol/l}$, of which 84 percent is bound to albumin, 15 percent

is tightly bound to α_2 -macroglobulin, and 1 percent to amino acids (Tapiero and Tew, 2003). Zinc binds to α_2 -macroglobulin tightly, preventing the exchange of zinc with other tissues. The major exchangeable zinc pool is serum albumin with two zinc-binding sites of high affinity (Tapiero and Tew, 2003). Transporter-mediated endocytosis of zinc and albumin has been suggested for zinc acquisition in endothelial cells (Rowe and Bobilya, 2000).

Gastrointestinal excretion is the main excretory route, and is calculated as 2.5–5.5 mg/d (Tapiero and Tew, 2003). Excretion occurs by (1) cell desquamation and transmucosal flux or (2) digestive secretion (mainly pancreatic) and is directly related to the total amount of zinc absorbed. If it is low, endogenous fecal zinc excretion is also low. In comparison with the amount of zinc loss through the gastrointestinal tract, renal losses tend to be low and remain constant over a wide range of intakes (King et al, 2001).

3.3 Mammalian zinc transporters

Mammalian zinc transporters can be classified into two major families, the ZIP (Zrt-, Irt-like Protein) family and CDF (Cation Diffusion Facilitator) family, by their similarities in topology, sequence, and functional properties (Reviewed by McMahon and Cousins, 1998; Reyes, 1996). The cation diffusion facilitator (CDF) family consists of transporters mainly responsible for zinc efflux or intracellular sequestration. CDF family members usually share a characteristic membrane topology including a six transmembrane domains and a cytoplasmic histidine-rich loop between membrane spanning regions IV and V, which may serve as a zinc-binding site. ZnT-1 was the first cloned and is associated with zinc efflux. It is found in all tissues examined, and, at least in some, ZnT-1 expression is regulated by dietary zinc intake. In enterocytes of the small intestine and renal tubular cells, ZnT-1 is localized to the basolateral membrane, suggesting an orientation that is consistent with zinc absorption/retention. ZnT-2 is

also an exporter and may be involved in zinc efflux or uptake into vesicles in intestine, kidney, and testis. ZnT-3 is involved in zinc uptake into vesicles in neurons and possibly in testis. ZnT-4 is also an exporter and is highly expressed in mammary gland and brain.

The human ZnT-like transporter 1, hZTL-1, shares 34% sequence identity to ZnT-1. Although it is classified in the CDF family, the topology of this protein is different from other members of this family. The number of predicted transmembrane domains is twelve for hZTL-1 whereas other members in the CDF family have six transmembrane domains. The functional activity of hZTL-1 is to facilitate zinc uptake. The localization of this protein in the apical membrane of Caco-2 cells suggests that it is a candidate transporter for zinc uptake (Cragg et al., 2002).

Members of the ZIP (ZRT-zinc regulated transporter, IRT-iron regulated transporter-like proteins) family, typically have eight membrane-spanning domains and an intracellular loop with a histidine rich region between transmembrane domains II and IV (Guerinot, 2000). This family of transporters is involved with zinc influx and uptake. Zip4, a transporter protein located at the apical surface of intestinal cells, has been found to facilitate carrier-mediated zinc uptake into the intestinal cell, The presence of Zip4 at the apical surface is responsive to dietary zinc, increasing with zinc deficiency and decreased during zinc sufficiency. Cell culture transfection studies indicate that the cellular zinc status regulates the level of endocytosis for the Zip4 transporter. Under normal zinc conditions, Zip4 recycles rapidly between the plasma membrane and a perinuclear endosomal compartment. During zinc deficiency or supplementation recycling slows. Zip4 shifts to the plasma membrane during deficiency and shifts to the intracellular endosomal vesicles during supplementation. The presence of the transporter at the plasma membrane corresponds with changes in zinc transport (Kim et al., 2004; Wang et al., 2004).

Transfection studies using cell culture identified Ztl1 as another zinc transport protein potentially involved in intestinal zinc uptake (Cragg et al., 2002). This transporter was also regulated by zinc. However, it was increases in zinc that induced the protein. The intracellular transport of zinc from the apical to basolateral intracellular surface for transport to the portal circulation is not known at this time. The extrusion of zinc from the cell into the portal circulation may occur through another zinc transporter protein, Znt-1 (Palmiter and Findley, 1995; McMahon and Cousins, 1997).

Non-specific metal-ion transporters such as divalent cation transporter 1 (DCT-1) also participate in the maintenance zinc homeostasis. This transporter, found in the crypts and villi of the duodenum, is regulated by iron, but exhibits transport activity for a number of trace elements including zinc (Gunshin et al., 1997). A schematic illustration of the postulated functions of these zinc transporters in a cell is shown in Figure 3.2. The mammalian zinc transporters are summarized in Table 3.2.

3.4 Zinc and Apoptosis

In the last three decades, substantial evidence *in vitro* and *in vivo* has accumulated linking zinc deficiency with a markedly increased susceptibility of cells and tissues to die by apoptosis (Reviewed by Maret, 2001). In nearly all examples of zinc deficiency-induced apoptosis *in vivo*, increased cell death appears to be a direct consequence of a lowering of intracellular levels of zinc in the affected tissues. In addition, numerous *in vitro* studies have shown that the depletion of intracellular zinc by culture of cells in zinc-depleted medium (Martin et al., 1991) or by treatment of cells with N,N,N',N'- tetrakis-2-pyridylmethylethylenediamine (TPEN) results in apoptosis (Zalewski et al., 1991, 1993). This is not atypical considering that the maintenance of

discrete subcellular pools of zinc is critical for the functional and structural integrity of cells, and contributes to a number of important biological processes (Truong-Tran et al., 2000).

Apoptosis is a regulated biological mechanism that involves a series of cytoskeletal, membrane, nuclear, and cytoplasmic changes required for the removal and deletion of superfluous, mutant or moderately damaged cells. This cellular demise occurs in two phases, consisting of (1) the biochemical signaling pathways that commit a cell to apoptosis and (2) the executional phase characterized by stereotypical morphological changes leading to cell death. Apoptosis results from the interaction between the initiating stimuli, which can be either physiological or injurious to the cell, and the factors determining the susceptibility of the cell to apoptosis (Truong-Tran et al., 2001).

Research suggests that there are likely two aspects to the anti-apoptotic mechanisms of action of zinc. Firstly, it limits the extent of damage induced by oxyradicals and other toxins, thereby suppressing some of the signaling pathways leading to apoptosis. Secondly, it directly affects some of the apoptotic regulators. A severe reduction in intracellular labile zinc may directly induce apoptosis while smaller decreases may simply render cells more vulnerable to apoptosis by other toxins (Tapiero and Tew, 2003).

3.5 Zinc Transport Studies

While numerous attempts have been made to determine that mechanism of zinc absorption from the gut, none have clearly defined the process. What is known, however, is that the higher the amount of dietary zinc intake, the lower the fractional rate of absorption, and vice versa (Payton et al., 1982). In rodent studies wherein the endogenous concentrations of zinc were controlled, the rate of zinc absorption was increased during zinc deficiency (Flanagan et al., 1983; Coyle et al., 2000). The amount of zinc absorbed can be controlled by the amount of zinc

administered; human subjects absorbed 56% of a 6 mg dose of zinc, but only 25% of a 60 mg dose (Payton et al., 1982). When the calculated amounts of zinc absorbed were plotted against the dose, a hypermetabolic relationship was revealed (Payton et al., 1982). This suggests that the absorption mechanism in the gut is saturable, and that the rate of zinc absorption in humans is regulated at the mucosal level.

3.5.1 Caco-2 Cell Line

Because it is difficult to control various parameters in the intestinal system of the whole animal, *in vitro* systems have been developed to define specific mechanisms of zinc uptake and transport. The Caco-2 is a colorectal carcinoma cell line, which upon differentiation loses its tumorigenic phenotype and displays characteristics of mature enterocytes, including brush borders with microvilli. Caco-2 cells can be used as an *in vitro* model to study zinc transport from the apical side to the basolateral side, as well as from the basolateral side to the apical side. The apical and basolateral sides of Caco-2 cells represent the luminal and blood sides, respectively, of the gastrointestinal tract *in vivo* (Raffaniello et al., 1992; Finley et al., 1995).

3.5.2 Nature of Studies

Zinc uptake mechanisms at the apical and basolateral membrane borders of Caco-2 cells were examined by Raffaniello et al. (1992), who demonstrated that separate mechanisms could be distinguished with respect to zinc uptake at the apical and basolateral membranes of Caco-2 cells. Caco-2 cells were grown until confluent and well-differentiated monolayers were formed. Labeled zinc was placed on the apical or basal side of the monolayer and its uptake by the cells, as well as its transport across the monolayer, were measured. Zinc uptake by the cells from the apical side was found to be a saturable process with a diffusional term at higher concentrations; it was not affected by metabolic inhibitors or potential zinc ligands. Zinc uptake from the

basolateral side was concentration dependent and was partially inhibited by ouabain and vanadate, suggesting that the (Na-K)-ATPase on the basolateral membrane is involved in the serosal uptake of zinc by the cell. Transport of zinc across the monolayers from the apical or basolateral compartment was concentration dependent and was not affected by metabolic inhibitors. Zinc transport from the basolateral side was greater than 2-fold greater than apical transport.

Caco-2 cells show an increased expression of mRNA corresponding to several zinc transporters with increased zinc concentration of nutrient medium (Ford, 2004). This is consistent with rodent models in which increased zinc availability increases expression of zinc transporters. The Caco-2 cell line has also been used to investigate the interaction between zinc and iron in intestinal mucosa, in terms of uptake (Yamaji et al., 2001). Cragg et al. (2002) reported the cloning of a novel human zinc transporter expressed at the apical membrane of Caco-2 cell model, whose sequence placed it in the CDF family but which has apparently different topology from the other cloned mammalian members. This protein was subsequently named hZTL1 (human ZnT- like transporter 1).

Reeves et al. (2001) used the Caco-2 cell line to further assess the adaptive response of the enterocytes to different concentrations of zinc. They exposed confluent, differentiated cells to high, but physiologic concentrations of media zinc for seven days. The rate of apical zinc uptake and transport of zinc across the monolayer were depressed. At the same time, however, the treatment enhanced these rates from the basolateral side of the cell. The study was concluded with the hypothesis that induced changes in transport rates by media zinc concentrations could involve the up-and/or down-regulation of zinc influx and efflux proteins.

3.6 Research Project

We investigated the effect of zinc deficiency on protein expression in the colon adenocarcinoma (Caco-2) cell-culture model. The aims of this research project include 1) the identification of expressed proteins of colon adenocarcinoma cells in response to incubation in zinc deficient medium, compared to control; and 2) to gauge the effectiveness of proteomics techniques (2DGE and MS) for investigating zinc metabolism.

Table 3.1: Dietary Reference Intake for Zinc

Nutrient	Life Stage Group	RDA/AI*	UL ^a
Zinc	Infants	(mg/d)	(mg/d)
	0- 6 mo	2*	4
	7-12 mo	3	5
	Children		
	1- 3 y	3	7
	4- 8 y	5	12
	Males		
	9- 13 y	8	23
	14- 18 y	11	34
	19- 30 y	11	40
	31-50 y	11	40
	50-70 y	11	40
	> 70 y	11	40
	Females		
	9- 13 y	8	23
	14- 18 y	9	34
	19- 30 y	8	40
31-50 y	8	40	
50-70 y	8	40	
> 70 y	8	40	
Pregnancy			
= 18 y	12	34	
19-30y	11	40	
31-50 y	11	40	
Lactation			
= 18 y	13	34	
19-30y	12	40	
31- 50 y	12	40	

NOTE: The table is adapted from the DRI reports, see www.nap.edu. It represents Recommended Dietary Allowances (RDAs) in **bold type**, Adequate Intakes (AIs) in ordinary type followed by an asterisk (*), and Tolerable Upper Intake Levels (ULs)^a. RDAs and AIs may both be used as goals for individual intake. RDAs are set to meet the needs of almost all (97 to 98 percent) individuals in a group. For healthy breastfed infants, the AI is the mean intake. The AI for other life stage and gender groups is believed to cover the needs of all individuals in the group, but lack of data prevent being able to specify with confidence the percentage of individuals covered by this intake.

^aUL = The maximum level of daily nutrient intake that is likely to pose no risk of adverse effects. Unless otherwise specified, the UL represents total intake from food, water, and supplements.

Special Considerations: Zinc absorption is lower for those consuming vegetarian diets than for those eating nonvegetarian diets. Therefore, it has been suggested that the zinc requirement for those consuming a vegetarian diet is approximately 2- fold greater than for those consuming a nonvegetarian diet.

Table 3.2 Mammalian zinc transporters

Transporters	Species	Distribution	Localization	Function
<i>CDF family</i>				
ZnT-1	rat/ mouse	Ubiquitous	Plasma membrane	Zinc efflux (Palmiter and Findley 1995)
ZnT-2	rat/ mouse	Intestine, kidney, testis	Intracellular vesicles	Zinc compartmentalization within cells (Palmiter et al. 1996a)
ZnT-3	mouse	Brain, testis	Synaptic vesicles	Sequester zinc into synaptic vesicles (Palmiter et al. 1996b)
ZnT-4	rat/ mouse /human	Mammary gland, heart, liver	Intracellular vesicles	Zinc compartmentalization within cells; Zinc deposition into milk (Huang and Gitschier 1997, Michalczyk et al. 2002, Murgia et al. 1999)
ZnT-5	human	Ubiquitous	Intracellular vesicles	Zinc transport into secretory granules of pancreatic beta cells (Kambe et al. 2002)

ZnT-6	Mouse/ human	Liver, kidney, brain, small intestine	Trans Golgi apparatus	Zinc transport between trans Golgi apparatus, and cytoplasmic vesicles (Huang et al. 2002)
hZTL-1	Human/ mouse	Ubiquitous	Plasma membrane	Zinc uptake (Cragg et al. 2002)
<i>ZIP family</i>				
hZIP1	human	Ubiquitous	Plasma membrane	Zinc uptake (Gaither and Eide 2001)
hZIP2	human	Prostate, uterine, epithelial cells	Plasma membrane	Zinc uptake (Gaither and Eide 2000)
hZIP4	human	Small intestine, kidney, colon	Plasma membrane	Zinc uptake (Wang et al. 2002)
<i>Nramp family*</i>				
DCT-1	rat	Ubiquitous	Plasma membrane	Divalent cation absorption (Gunshin et al. 1997)

* Nramp family: natural-resistance-associated macrophage protein family

Figure 3.1: Factors Predisposing to Zinc Deficiency**Increased Requirement**

Burns
 Chronic Infection
 Dermatological Disorders
 Infantile and Adolescent Growth Spurts
 Malignancy
 Pregnancy/Lactation
 Surgery

Inadequate Dietary Intake

Alcoholism
 Anorexia
 Chronic Uremia
 Low Socioeconomic Status
 Poor Food Choices
 Protein-Calorie Malnutrition
 Restricted Protein Diets
 Synthetic Diets
 Total Parenteral Diets without Added Zinc
 Vegetarianism

Decreased Absorption

Celiac Disease/Other Enteropathies
 Chronic Inflammatory Bowel Disease
 Diet High Phytate, Alcohol, Chelating Agents
 Drugs-Corticosteroids
 Acrodermatitis Enteropathica
 Gastrointestinal Dysfunction
 Geophagia
 Immaturity of Absorptive Mechanisms
 Liver Disease/ Alcoholic Cirrhosis

Increased Losses

Alcohol/ Alcoholism/ Alcohol Cirrhosis
 Diabetes Mellitus
 Dialysis
 Diarrheal Fluid Loss
 Drugs- Thiazide Diuretics, Penicillamine, Chelating Agent
 Gastrointestinal Infestation
 Muscular Dystrophy
 Muscle Catabolism
 Post Surgery/Infection/Trauma
 Proteinuria
 Renal Disease
 Sickle-cell Disease
 Starvation
 Viral Hepatitis

**ZINC
 DEFICIENCY**

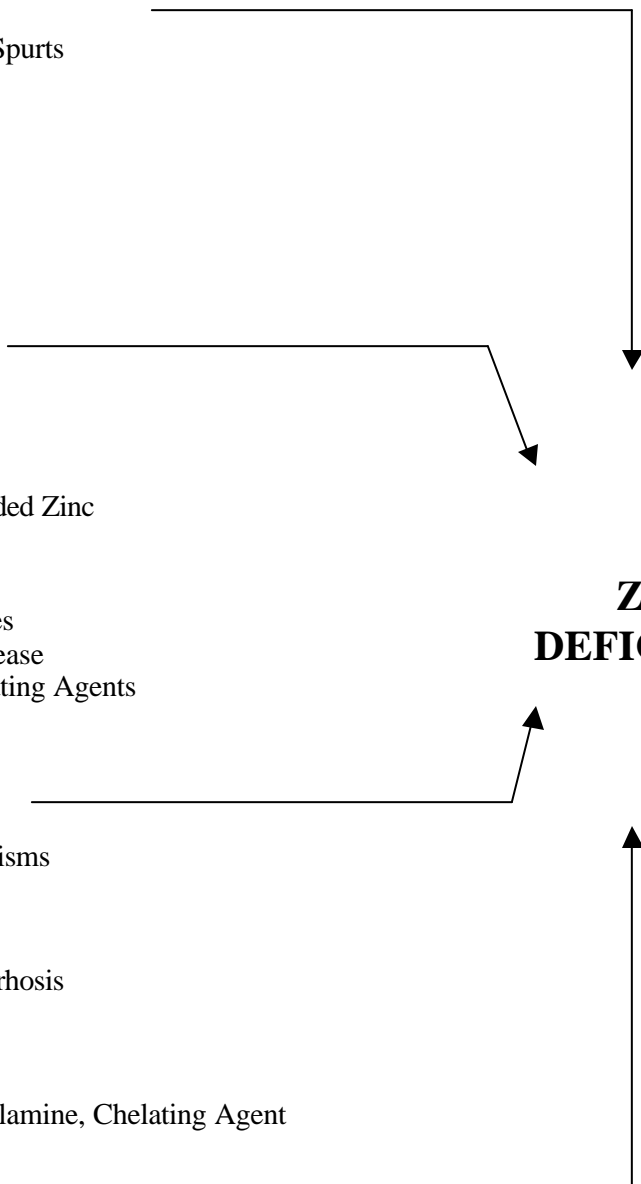
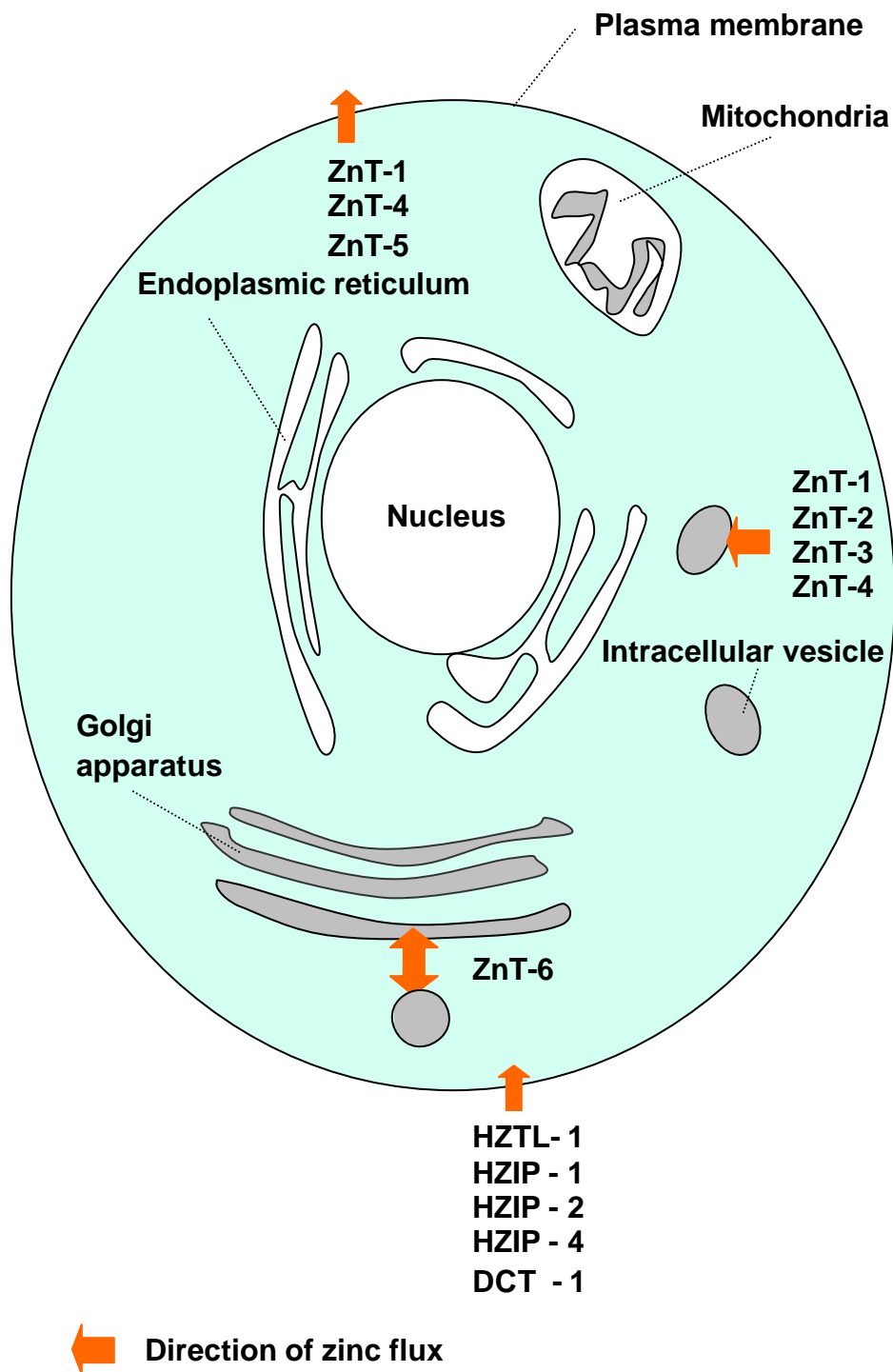


Figure 3.2: Schematic illustration of zinc transporters in a cell.



CHAPTER 4

ZINC DEFICIENCY AFFECTS PROTEIN EXPRESSION IN CACO-2 CELLS¹

¹ Erica Renee Berelc, Michael Frank Mouat, Arthur Grider. Revised version to be submitted to Proteome Science.

Abstract

Zinc (Zn) is an essential nutrient that is necessary for a wide range of cellular processes. It is essential for the structural and functional integrity of cells, and plays a pivotal role in the control of gene expression. Though the mechanisms for intestinal Zn absorption have not been clearly identified, it is generally accepted that 1) the absorption of zinc is concentration dependent, i.e., the higher amount of dietary zinc intake, the lower the fractional rate of absorption and vice versa, 2) the absorption mechanism in the gut is saturable, and 3) the rate of zinc absorption in humans is regulated at the mucosal level. We investigated the effect of zinc deficiency on protein expression in a colon adenocarcinoma (Caco-2) cell-culture model, using two-dimensional gel electrophoresis (2DGE) in conjunction with mass spectrometry (MS). Caco-2 cells were grown on membrane inserts in minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS) for 20 days. At day 14, the cells were maintained in either fresh normal MEM (12 μM Zn) or medium containing 20% dialyzed FBS (0.35 μM Zn) for 7 days. The total cell lysates (2 mg) were separated using a large format 2DGE system. The gels were stained with 0.1% Coomassie Blue R-250. Following destaining, the gels were analyzed by Phoretix 2D Advanced software (v6.01). Approximately 400 spots were detected between the two gel preparations. The differentially expressed proteins were excised, digested with trypsin, and analyzed by mass spectrometry. Identified proteins that responded to zinc status in Caco-2 cells could be sorted into 8 functional categories. Identified groups included calcium binding (3), chaperones (3), signal transduction (4), energy (9), nuclear or DNA/RNA binding (6), channel (1), structural (8), and serum (4).

Background

Zinc is an essential trace mineral present in all eukaryotic organisms, with cofactor functions in a large number of proteins of intermediary metabolism, hormone secretion pathways, and immune defense mechanisms (Vallee and Auld, 1990). It is required for more than 300 enzymes belonging to all six enzyme classes, including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee and Falchuk, 1993). In these zinc proteins, the major role of the zinc ion can be catalytic, cocatalytic or structural (Vallee and Falchuk, 1993; Reviewed by Mcall et al, 2000). On the basis of its multiple biological functions, zinc deficiency causes a wide variety of symptoms including impaired growth, anorexia, diarrhea, dermatitis, alopecia, and abnormal development (Prasad, 1998).

In mammals, zinc is absorbed in the small intestine through the apical surface of enterocytes (Lonnerdal, 1989). Though the molecular mechanisms involved in the transepithelial transport of zinc are still poorly understood, we do know that the absorption of zinc is regulated. The higher the amount of dietary zinc intake, the lower the fractional rate of absorption, and vice versa (Payton et al., 1982). This process seems to be adaptable because the amount of zinc in mucosal cells and blood is kept at near normal concentrations following continuous consumption of high dietary zinc (Reeves, 1995).

Reeves et al. (2001) used Caco-2 cells, an *in vitro* cell culture model that exhibits similar structural and functional characteristics of small intestinal enterocytes, to assess the adaptive response of the enterocytes to different concentrations of zinc. They found that the rate of apical zinc uptake and transport of zinc across the monolayer were depressed when cells were exposed to high, but physiological concentrations of zinc. The induced changes in transport rates by

media zinc concentrations could involve the up-and/or down-regulation of zinc influx and efflux proteins (Reeves et al. 2001).

We investigated the effect of zinc deficiency on protein expression in Caco-2 cells using two-dimensional gel electrophoresis (2DGE) along with mass spectrometry (MS). These methods allow the qualitative and quantitative comparison of protein expression of different samples, i.e. control and treatment. The appearance or disappearance of specific protein spots indicates differential protein expression, while the degree of spot intensity provides quantitative information about protein expression levels.

In this study, we show that the exposure of confluent, differentiated cells to zinc-deficient medium (0.35 μM Zn) for 7 days, altered the expression of numerous proteins. The proteins whose expression was affected by zinc deficiency functioned in numerous biochemical and physiological pathways including cytoskeletal, signal transduction, calcium-binding, and energy metabolism.

Results

To deplete the cells of intracellular zinc, Caco-2 cells were incubated in zinc-deficient medium (MEM + 20% dialyzed FBS; 0.35 μM Zn) for 7 days following differentiation. In control experiments, undialyzed FBS was added to MEM (12 μM Zn) for 7 days following differentiation. The concentrations of zinc in the media were found using an atomic absorption spectrophotometer.

To identify proteins responsive to a low intracellular zinc concentration, we used 2DGE coupled with mass spectrometry. The total cell lysates (2 mg) were separated using a large format 2DGE system; gels were stained with 0.1% Coomassie Blue R-250. Following

destaining, the gels were analyzed by Phoretix 2D Advanced software (v6.01). Protein spots were excised manually, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry.

Figures 3.3 and 3.4 represent 2DGE images of the control gel and deficient gel, respectively. Gels were stained with Coomassie Blue R-250. Approximately 400 spots were detected between the two gel preparations. Six proteins were identified as being differentially expressed in the control gel. The identity and functions of these proteins are summarized in Table 4.1 and Figure 4.1. Incubation of Caco-2 cells in Zn-deficient medium (0.35 μ M Zn) for 7 days following differentiation resulted in the differential expression of 47 proteins. The identity and functions of these proteins are summarized in Table 4.2 and Figure 4.2.

Identified proteins that responded to zinc status in Caco-2 cells could be sorted into 8 functional categories. Identified groups included calcium binding (3), chaperones (3), signal transduction (4), energy (9), nuclear or DNA/RNA binding (6), channel (1), structural (8), and serum (4). No zinc transporters were identified at this time.

Discussion

In the present study, we used the human colon adenocarcinoma cell line Caco-2 to identify proteins expressed under conditions of zinc deficiency. The results of this method indicated that the depletion of intracellular zinc altered the expression of numerous proteins. The proteins whose expression was affected by zinc deficiency functioned in numerous biochemical and physiological pathways including cytoskeletal, signal transduction, calcium-binding, and energy metabolism. Of particular interest are those proteins involved in cell cycle regulation. These include the heat shock proteins, creatine kinase, structural proteins (keratins, actin, and tubulin), glyceraldehyde 3-phosphate dehydrogenase, and the 14-3-3 family.

Many of the 47 proteins that were found to be differentially expressed due to incubation in zinc deficient medium are associated with apoptosis. Apoptosis is a regulated biological mechanism that involves a series of cytoskeletal, membrane, nuclear, and cytoplasmic changes required for the removal and deletion of superfluous, mutant or moderately damaged cells. Over the span of the last three decades, substantial evidence *in vitro* and *in vivo* has accumulated linking zinc deficiency with a markedly increased susceptibility of cells and tissues to die by apoptosis (Reviewed by Truong-Tran et al., 2000). Numerous *in vitro* studies have shown that the depletion of intracellular zinc by culture of cells in zinc-depleted medium (Martin et al., 1991) or by treatment of cells with N,N,N',N'-tetrakis-2 pyridylmethylethylenediamine (TPEN) results in apoptosis (Zalewski et al., 1991; 1993).

One of the morphological changes observed during apoptosis is the loss of plasma-membrane asymmetry, associated with phosphatidylserine translocation from the inner to the outer monolayer of the membrane. Annexins (Spots 32, 41) comprise a family of calcium- and phospholipid-binding proteins. Annexins bind to phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol, which are known to be rich in the inner leaflet of plasma membrane and hardly appear on cell surface, in contrast to phosphatidylcholine and sphingomyelin, which are major components of the outer leaflet of plasma membrane (Mollenhauer et al., 1997). It is reasonable to assume that the proposed functions of intracellular annexins include regulation of phospholipase A2 activity due to sequestration of substrate phospholipids, and involvement in vesicular transport and trafficking, endocytosis, and exocytosis (Mollenhauer et al., 1997). Shifts in subcellular locations (from the cytosol to membrane) are observed on some intracellular annexins, suggesting the active movement of annexins corresponding to dynamic lipid vesicle transport. Annexins are exported from cytosol to the outside of cells across the plasma

membrane by unknown mechanisms. Although annexins lack hydrophobic signal peptides, secretion and expression on cell surface experienced by some annexins are evident in some cell types. After being exported outside of cells, some annexins have been shown to function as receptors for extracellular proteins; annexin V binds to collagen and annexin II binds to tissue plasminogen activator and tenascin.

S100A5 (Spot 40) is a novel member of the EF-hand superfamily of calcium-binding proteins that is poorly characterized at the protein level. EF-hands are calcium-binding motifs that occur at least in pairs. Links between disease states and genes encoding EF-hands, particularly the S100 subclass, are emerging. Each motif consists of a 12-residue loop flanked on either side by a 12-residue alpha-helix. EF-hands undergo a conformational change upon binding calcium ions. The S100A5 dimer binds two Zn^{2+} ions with a $[Zn^{2+}]_{0.5}$ of 2 μM . Zn^{2+} binding does not influence Ca^{2+} binding, but it is likely that Zn^{2+} enhances the interaction of the protein with targets. S100A5 is an unusual member of the S100 protein family in that it has a high affinity for Ca^{2+} , Zn^{2+} , and Cu^{2+} and that it induces distinct structural changes (Schafer et al., 2000).

Zinc participates in the maintenance of the normal structure and function of membranes. Ezrin (Spot 17) is a protein enriched in cell surface microvilli; its function is to provide a regulated linkage between the actin cytoskeleton and the plasma membrane. Tubulin (Spot 26) is the protein that polymerizes into long chains or filaments that form microtubules, hollow fibers which serve as a skeletal system for living cells. Microtubules have the ability to shift through various formations, which is what enables a cell to undergo mitosis or to regulate intracellular transport. Actins (Spots 14, 30) are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. In mammalian nonmuscle cells, 2 classes of actin are recognized on isoelectric focusing gels: beta and gamma. These 2

isoforms differ by 4 amino acid substitutions at the conserved NH₂-end of the molecule. They are coexpressed in nonmuscle cells (Kedes et al., 1985).

The Galectins (Spots 37, 38) are lectins that recognize and interact with beta-galactoside moieties. Galectin-3 regulates a number of biological processes, including embryogenesis, inflammatory responses, cell progression and metastasis. Galectin-3 is normally expressed in epithelia of a variety of tissues, including colon and endometrium, and in various inflammatory cells, including macrophages. Galectin-3 can function intracellularly, in controlling cell cycle and preventing T-cell apoptosis, and also extracellularly, in activating various cells, including monocytes/macrophages, mast cells, neutrophils, and lymphocytes. One of its more interesting proposed functions is as an inhibitor of apoptosis (Kashio et al., 2003). Galectin-3 shares an NWGR motif with Bcl-2 which has been shown to be an important region in Bcl-2 for suppression of apoptosis.

Heat shock proteins (Spots 7, 9) are a family of cellular proteins characterized by their up-regulation in response to stress and the presence of a weak ATPase activity (Richter et al., 2001). They were initially described as chaperones that facilitate the folding of other proteins. However, recent studies (Aligue et al., 1994; Cutforth and Rubin, 1994; Nathan and Lindquist, 1995; Pratt and Toft, 1997) indicate that heat shock proteins also play a role in cytoprotection, anti-apoptosis, and signal transduction by forming complexes with nuclear receptors and protein kinases, such as Raf, Src, p21 *ras*, p53, and Rb. Hsp90 is constitutively expressed in the cytoplasm of cells, and it has been shown to be secreted from cells in response to stress (Liao et al. 2000). It should be noted that Hsp90 is a generic term used to describe two isoforms whose actual M_r s are 86 and 84 kDa termed Hsp90.

Young et al. (2003) showed that the cytosolic chaperones HSP90 and HSP70 dock onto a specialized tetratricopeptide (TPR) domain in the import receptor TOMM70 at the outer mitochondrial membrane. This interaction served to deliver a set of preproteins to the receptor for subsequent membrane translocation dependent on the HSP90 ATPase. Disruption of the chaperone/TOMM70 recognition inhibited the import of these preproteins into mitochondria. Young et al. (2003) proposed a mechanism in which chaperones are recruited for a specific targeting event by a membrane-bound receptor.

One of the most interesting proteins found to be upregulated due to incubation in zinc-deficient medium, was the voltage-dependent anion-selective channel protein (Spot 36). Voltage-dependent anion-selective channel proteins (VDACs) are pore-forming proteins found in the outer mitochondrial membrane of all eukaryotes and in brain postsynaptic membranes. VDAC channels are thought to regulate the mitochondrial metabolism by regulating the flux of metabolites across the mitochondrial outer membrane. VDACs are involved in different cellular events like the induced release of cytochrome *c*, which constitutes an early step in apoptosis (Shimizu, 1999). During transduction of an apoptotic signal into the cell, there is an alteration in the permeability of the membranes of the cell's mitochondria, which causes the translocation of the apoptogenic protein cytochrome *c* into the cytoplasm, which in turn activates death-driving proteolytic proteins known as caspases (OMIM 147678). The BCL2 family of proteins, whose members may be antiapoptotic or proapoptotic, regulates cell death by controlling this mitochondrial membrane permeability during apoptosis (Benz et al., 1988; Rostovtseva and Colombini, 1997).

Shimizu et al. (1999) created liposomes that carried the mitochondrial porin channel VDAC to show that the recombinant proapoptotic proteins Bax (OMIM 600040) and Bak (OMIM

600516) accelerate the opening of VDAC, whereas the antiapoptotic protein BCLXL (OMIM 600039) closes VDAC by binding to it directly. Bax and Bak allow cytochrome c to pass through VDAC out of liposomes, but passage is prevented by BCLXL. In agreement with this, VDAC1-deficient mitochondria from mutant yeast did not exhibit a Bax/Bak-induced loss in membrane potential and cytochrome c release, both of which were inhibited by BCLXL. Shimizu et al. (1999) concluded that the BCL2 family of proteins binds to the VDAC in order to regulate the mitochondrial membrane potential and the release of cytochrome c during apoptosis.

14-3-3 proteins (Spots 42-44) modulate, by their action with more than 100 binding partners, the action of proteins that are involved in cell cycle and transcriptional control, signal transduction, intracellular trafficking, and regulation of ion channels. There are seven known mammalian isotopes (β , γ , ϵ , ζ , η , θ , and ι). Note: α has been renamed β ; and δ has been renamed γ . The control that 14-3-3 proteins exert on the subcellular localization of their binding partners, as well as their ability to stimulate protein-protein interactions is crucial for apoptosis.

Glyceraldehyde 3- Phosphate Dehydrogenase (GAPD; Spot 33) catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD). GAPD has also been shown to bind to RNA, ATP, calyculin, actin, tubulin and amyloid precursor protein.

Human colonic adenocarcinoma cells (Caco-2 cells) are valuable *in vitro* tools for studies related to intestinal cell function and differentiation. Caco-2 cells are able to differentiate into small intestine-like enterocytes; they polarize when plated on Transwells® membrane supports, allowing for the study of apical to basolateral and basolateral to apical zinc transport. Reeves et al. (2001) used the Caco-2 cell line to investigate the adaptive response of enterocytes to high,

but physiologic, concentrations of zinc. They exposed confluent, differentiated cells to 5 or 25 $\mu\text{mol Zn/L}$ for seven days. Zinc uptake and transport, as well as the release of zinc to the apical and basolateral sides, were measured in both apical and basolateral directions using ^{65}Zn . The rate of apical zinc uptake and transport of zinc across the monolayer were depressed in cells exposed to 25 $\mu\text{mol Zn/L}$. At the same time, however, the treatment enhanced these rates from the basolateral side of the cell. The study was concluded with the hypothesis that induced changes in transport rates by media zinc concentrations could involve the up-and/or down-regulation of zinc influx and efflux proteins.

The activity of membrane transporters that mediate zinc uptake and efflux as well as intracellular zinc sequestration are believed to be central to cellular zinc homeostasis (King et al. 2002). Several zinc transporters have been identified to date (McMahon and Cousins 1998). Mammalian zinc transporters can be classified into two major families, the ZIP (Zrt-, Irt-like Protein) family and CDF (Cation Diffusion Facilitator) family, by their similarities in topology, sequence, and functional properties (Reviewed by McMahon and Cousins 1998; Reyes 1996). King et al. (2002) cloned a novel zinc transporter, termed hZTL-1, expressed at the apical membrane of the Caco-2 cell line. The functional activity of hZTL-1 is to facilitate zinc uptake. The localization of this protein in the apical membrane of Caco-2 cells suggests that it is a candidate transporter for zinc uptake (Cragg et al., 2002). We did not identify any zinc transporters at this time.

Conclusion

While this investigation was based on the study by Reeves et al. (2001), the procedures we used were somewhat different. We investigated the effect of zinc deficiency on protein expression in Caco-2 cells using two-dimensional gel electrophoresis (2DGE) along with mass

spectrometry (MS). These methods allow the qualitative and quantitative comparison of protein expression of different samples, i.e. Caco-2 cells grown in zinc adequate versus zinc deficient media, at the time of isolation. These studies, therefore, provide a more complete picture of the adaptive response that the cell or tissue mounts following that treatment.

In addition to its diverse role in many physiological systems, zinc has been shown to be an important regulator of apoptosis. Subtle changes in cellular zinc content and localization have been reported to have profound effects on cell metabolism and function. In humans, zinc deficiency negatively affects the epidermal, central nervous, immune, gastrointestinal, skeletal, and reproductive systems.

Our conclusion is that the Caco-2 model is a good cell culture model and that proteomics techniques are powerful tools, for the study of the effect of zinc deficiency on protein expression. Future studies will focus on the role zinc plays in expression of proteins associated with cell cycle regulation, especially apoptosis.

Materials and Methods

Human colon adenocarcinoma cells (Caco-2 line) were obtained from the American Type Culture Collection, Manassas, VA, at passage 20. The colon adenocarcinoma cells had been isolated from a Caucasian male, aged 72 years (HTB-37). Transwell® tissue culture treated polycarbonate membrane polystyrene plates (24mm diameter, 0.4 µm pore size) were purchased from Corning Incorporated, Corning, NY. Cell culture media and chemicals were obtained from Sigma Chemical Co., Summit Biotechnology, and/or Gibco Laboratories. Zinc concentrations in media were measured with an atomic absorption spectrophotometer.

Cell Culture

Cells were seeded (58,000 cell/cm²) onto membrane inserts that hung inside the chambers of six-well plates. Two mL of growth medium were placed inside the insert (apical side; AP) and 4 mL were placed inside each chamber (basolateral side; BL). The growth medium was composed of Minimal Essential Medium (MEM) with 2mM L-glutamine and Earle's balanced salts to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, fetal bovine serum, 20%. The media was replaced at three-day intervals throughout the growth, differentiation, and experimental periods. The dialysis medium consisted of the following: 150 mM NaCl, 100 mM KBr, 10 mM EDTA, pH 7.2. Two hundred ml of fetal bovine serum was dialyzed against 4 l of dialysis medium for 24 h at 4 C with gentle stirring. The medium was then replaced with 4 l fresh dialysis medium and the dialysis continued for another 24 h at 4 C. Replacement of medium followed by a further 24 h dialysis was repeated two more times. The dialyzed serum was retained for addition to the cell culture medium.

Preparation of total cell lysate

The wells were rinsed three times (5 minutes each time) with 4 mL ice-cold wash buffer (2.7 mM KCl, 1.5 mM KH₂PO₄, 136 mM NaCl, 8.1 mM Na₂HPO₄·7 H₂O, 18 meg Ohm water). After removal of the final volume of wash buffer, 30 µL of boiling sample buffer 1 (0.3% SDS, 200 mM DTT, 28 mM Tris HCl, 22 mM Tris base, 18 meg Ohm water) was added to each well, the cells scraped together into the buffer and the cell lysate transferred into 1.5- mL microfuge tubes. The cell lysate was heated for five minutes at 100°C, chilled on ice for 5 minutes, and 9 µL of sample buffer 2 (24 mM Tris base stock, 476 mM Tris HCl stock, 50 mM MgCl₂ stock, 1.0 mg/mL DNase 1, 0.25 mg/mL RNase A (10 mg/mL) was added. After incubation on ice for 8 minutes, the cellular proteins were precipitated by the addition of acetone to 80% v/v and

incubation on ice for twenty minutes. The microfuge tubes were then centrifuged at 12,000 X g for ten minutes at 4°C, the supernatant discarded, and the pellet dried at room temperature for five minutes. The pellet was resuspended in 240 µL of IPG Sample Buffer (7M Urea, 2M Thiourea, 4% CHAPS, 2% Pharmalyte (3-10), 1% DTT), and an aliquot was removed for assaying the protein concentration. The remainder was stored at -80°C. The protein concentration was determined using the Bradford method (Bradford 1976, Bio-Rad, Hercules, CA).

2DPAGE- first dimension isoelectric focusing (IEF)

The proteins from Caco-2 cells grown in normal MEM (12 µM Zn) and Caco-2 cells grown in MEM containing 20% dialyzed FBS (0.35 µM Zn) were separated by two-dimensional gel electrophoresis. Samples were mixed with rehydration buffer (6M Urea, 2M Thiourea, 2% CHAPS, 0.5% Pharmalyte (3-10), 0.4% DTT) to a total volume of 300 µL at a concentration of 2 mg/mL and loaded onto a precast immobilized pH gradient (IPG) gel strip (Bio-Rad). They were then overlaid with mineral oil and allowed to rehydrate under passive condition for 12-16 hours at 20°C. First dimensional IEF was performed for 60,000 volt-hours.

2DPAGE- second dimension

After IEF, the IPG strips were equilibrated with 6 M urea, 30% glycerol, 2% SDS, 1.55 M Tris, 2 mM TBP, for 25 min at room temperature, then loaded onto large format (22 cm X 22 cm X 1 mm) 12% acrylamide slab gels. The gels were run at 20 W/gel at 4°C until the dye front reached within 1 cm from the bottom of the gel. The cathode buffer contained 50 mmol/L Tris base, 384 mmol/L glycine and 6.9 mmol/L sodium dodecyl sulfate. The anode buffer contained 25 mmol/L Tris base, 192 mmol/L glycine and 3.5 mmol/L sodium dodecyl sulfate. The separated proteins were visualized by Coomassie blue staining.

Staining

After running the second dimension, the gels were stained overnight in 1L 40% methanol/ 0.1% Coomassie Brilliant Blue R-240/ 7% acetic acid/ 52% water. They were then washed with Destain I (40% methanol, 7% acetic acid, 53% water) for 1 hour, and Destain II (5% methanol, 7% acetic acid, 88% water) for 30 minutes. The gels were temporarily kept in a solution of 7% acetic acid/ 93% water and stored at 4°C in zip lock bags.

Analysis

The spots that were differentially expressed or up regulated were excised from six gels. The gel pieces were sent to the Proteomics Resource Facility (University of Georgia, Athens, GA) for in-gel digestion and amino acid sequencing: Two mm gel plugs were picked, washed, digested with trypsin; the resulting peptides were extracted and spotted using the Spot Handling Workstation (Amersham Biosciences). Briefly, plugs were washed twice with 50 mM ammonium bicarbonate/50% methanol for 20 minutes at room temperature. Plugs were washed with 75% acetonitrile for 20 minutes at room temperature and dried at 40°C for 10 minutes. Plugs were then incubated with 140 ng sequencing grade trypsin (Promega) at 37°C for 1 hour. Peptides were extracted twice with 50% acetonitrile/0.1% TFA for 20 minutes at room temperature. Approximately 25% of the resulting peptides were spotted with partially saturated *a*-cyano-4-hydroxy-cinnamic acid (Sigma). Mass spectrometry (MS) data were acquired on the 4700 Proteomics Analyzer (Applied Biosystems) using standard acquisition methods. MS spectra were calibrated using two trypsin autolysis peaks (1045.45 and 2211.096 m/z). Mass lists were submitted to NCBI Inr and SwissProt using Mascot (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) (Human). Identifications were cross-examined using mass accuracy, molecular weight, and pI.

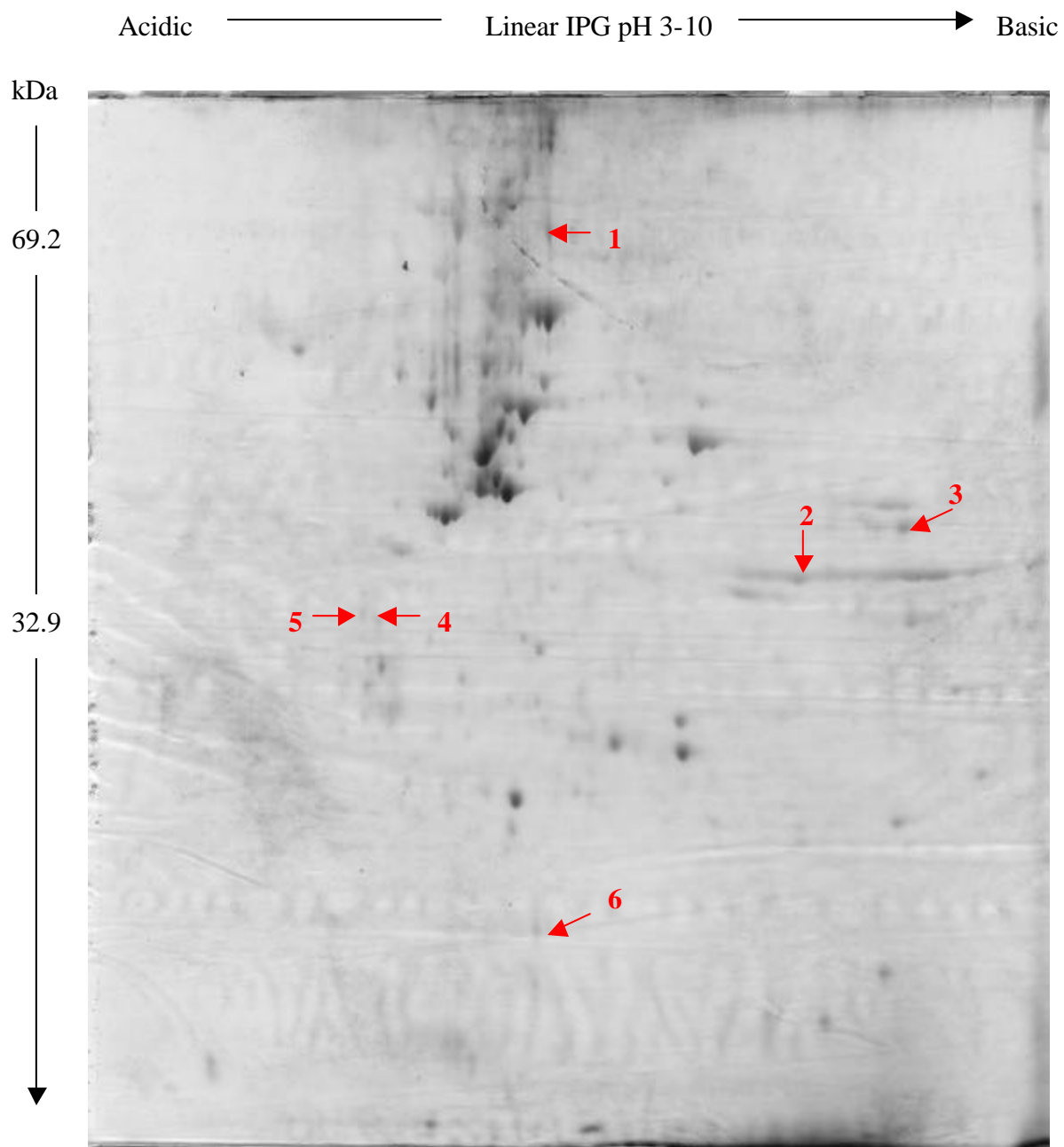


Figure 4.1: 2D image of proteins from Caco-2 cells grown in fresh normal MEM (12 μ M Zn). Protein extracts were separated on pH 3-10 linear IPG strips. This was followed by electrophoresis on 12.5% SDS-polyacrylamide gels, as stated in Materials and Methods. The gel was stained with Coomassie blue. Differential protein expression between dietary groups was analyzed by Phoretix software.

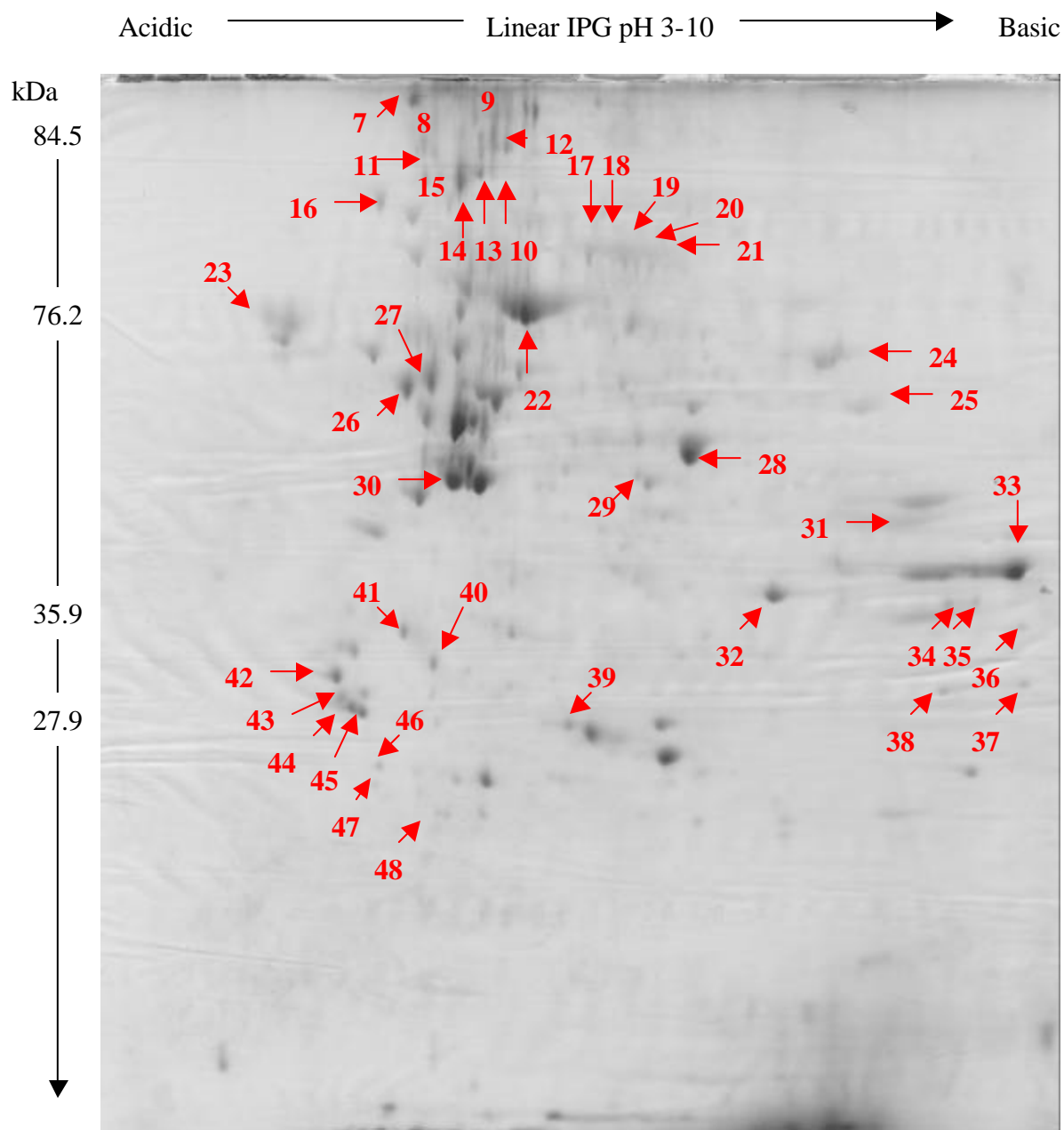


Figure 4.2: 2D image of proteins from Caco-2 cells incubated in zinc-deficient MEM (0.35 μ M Zn) for 7 days following differentiation. Protein extracts were separated on pH 3-10 linear IPG strips. This was followed by electrophoresis on 12.5% SDS-polyacrylamide gels, as stated in Materials and Methods. The gel was stained with Coomassie blue. Differential protein expression between dietary groups was analyzed by Phoretix software.

Table 4.1: Overview of Caco-2 proteins expressed in response to growth in normal MEM (12 μ M Zn).*

Function Category ^a	Spot no ^b	Annotation and/or Homologue Proteins ^c	Accession # ^d	MW (kDa) ^e
Energy	3	Fructose-Bisphosphate Aldolase (EC 4.1.2.13)	sp P04075 ALFA_	39264.3
Structural	4	Tropomyosin, Fibroblast Isoform TM3	sp P09494 TPMF_	32855.8
Serum	1	Serum Albumin Precursor	sp P02769 ALBU_	69248.4
Proteinase	6	Acrosin Precursor (EC 3.4.21.10)	sp P29293 ACRO_	48248.6

*Incubated in normal MEM before and following differentiation; identified after in-gel digestion with trypsin and MALDI-TOF MS.

^a Function of encoded protein.

^b Refers to the proteins labeled in Figure 4.1.

^c Name of identified protein.

^d SwissProt.

^e Molecular Weight.

Note: Numbers 2 and 5 are unidentified at this time.

Table 4.2: Overview of Caco-2 proteins expressed in response to intracellular zinc depletion.*

Function Category ^a	Spot no ^b	Annotation and/or Homologue Proteins ^c	Accession # ^d	MW (kDa) ^e
Calcium Binding	32	Annexin II (Lipocortin II)	sp P07355 ANX2_	38448.8
	41	Annexin V (Lipocortin V)	sp P08758 ANX5_	35783.4
	40	S100 Calcium-Binding Protein A5	sp P33763 S105_	12804.3
Chaperones	7	Heat Shock Protein HSP 90-Alpha (HSP 86)	sp P07900 HS9A_	84489.6
	9	Heat Shock Cognate 71 KD Protein	sp P11142 HS7C_	70854.2
	16	Endoplasmic Precursor (94 KD Glucose-Related Protein)	sp P14625 ENPL_	92411.3
Signal Transduction	42	14-3-3 Protein Epsilon (Mitochondrial Import Stimulation Factor L Subunit)	sp P42655 143E_	29155.4
	43	14-3-3 Protein Tau (14-3-3 Protein Theta)	sp P27348 143T_	27746.8
	45	14-3-3 Protein Beta/ Alpha (Protein Kinase C Inhibitor Protein 1)	sp P31946 143B_	27933.8
	44	14-3-3 Protein Zeta/ Delta (Protein Kinase C Inhibitor Protein 1)	sp P29312 143Z_	27727.7
Energy	10	Creatine Kinase, B Chain (EC 2.7.3.2)	sp P12277 KCRB_	42617.3
	24	Pyruvate Kinase, M2 Isozyme (EC 2.7.1.40)	sp P14786 KPY2_	57746
	25	ATP Synthase Alpha Chain, Mitochondrial Precursor (EC 3.6.1.34)	sp P25705 ATPA_	59713.6
	28	Alpha Enolase (EC 4.2.1.11)	sp P06733 ENOA_	47008.3
	33	Glyceraldehyde 3- Phosphate Dehydrogenase	sp P04406 G3P2_	35899.4
	39/46	ATP Synthase F Chain, Mitochondrial (EC 3.6.1.34)	sp Q28851 ATPK_	10159.5
	23	Tyrosine Protein Kinase BTK (EC 2.7.1.112)	sp Q06187 BTK_H	76232.4
	31	Fructose-Bisphosphate Aldolase (EC 4.1.2.13)	sp P04075 ALFA_	39264.3
Nuclear or DNA/ RNA Binding	15	Transitional Endoplasmic Reticulum ATPase	sp P03974 TERA_	89232.7

	29	Elongation Factor Tu, Mitochondrial Precursor (P43)	sp P49411 EFTU_	49510.2
	34/35	Heterogeneous Nuclear Ribonucleoproteins A2/B1	sp P22626 ROA2_	37406.7
	37/38	Galectin 3 (Galactose Specific Lectin 3)	sp P17931 LEG3_	26041
Channel	36	Voltage Dependent Anion Selective Protein 1	sp P21796 POR1_	30622.5
Structural	8/27	Tubulin Alpha-6 Chain	sp P05216 TBA6_	49877.5
	12	Keratin, Type II Cytoskeletal 8	sp P05787 K2C8_	53510.1
	11	Keratin, Type I Cytoskeletal 19	sp P08727 K1CS_	44079.1
	14	Actin, Cytoplasmic 1(Beta- Actin)	sp P02570 ACTB_	41709.7
	17	Ezrin (P81)	sp P15311 EZRI_	69224.7
	26	Tubulin Beta-5 Chain	sp P05218 TBB5_	49639
	30	Actin, Cytoplasmic 2 (Gamma-Actin)	sp P02571 ACTG_	41765.8
Serum	22	Serum Albumin Precursor	sp P02769 ALBU_	69248.4
	18-20	Serotransferrin Precursor (Siderophilin)	sp Q29443 TRFE_	77702.7

*Incubated in zinc deficient medium following differentiation; identified after in-gel digestion with trypsin and MALDI-TOF MS.

^a Function of encoded protein.

^b Refers to the proteins labeled in Figure 4.2.

^c Name of identified protein.

^d SwissProt.

^e Molecular Weight.

Note: Numbers 13, 21, 47, and 48 are unidentified at this time.

CHAPTER 5

SELENIUM

Selenium (Se) is an essential micronutrient in the diet of humans and other mammals. Ever since its biological role in humans was first characterized, evidence for its increasing scope and importance to human health has been rapidly accumulating.

4.1 Importance of selenium as a nutrient

The importance of selenium in the human diet was discovered in 1979 when a group of Chinese scientists correlated selenium deficiency with Keshan disease (Reviewed by Beck et al., 2003). Keshan disease, a cardiomyopathy that leads to cardiogenic shock and, in some cases, congestive heart failure, was first noticed in China during the early 1930's (Gu, 1983; Keshan Disease Research Group, 1979). Individuals living in areas with selenium-poor soils seemed susceptible to development of Keshan disease. Furthermore, affected individuals had low dietary intakes of selenium, reflected in low serum and hair levels of selenium. Supplementation of individuals with sodium selenite could completely prevent the development of Keshan disease, which provided evidence that a deficiency in selenium was the cause of the malady (Burk and Levander, 1999). However, the incidence of Keshan disease fluctuated seasonally and not every selenium-deficient individual developed the disease. Taken together, these findings by scientists in China suggested that an infectious cofactor was required along with a deficiency in selenium for the development of Keshan disease (Keshan Disease Research Group, 1979).

These discoveries led to expansive research investigating further unknown roles of selenium within the human body. The massive influx of selenium research led to the establishment of dietary recommendations from the World Health Organization (WHO). In

accordance with the WHO, a Recommended Daily Allowance (RDA) was established for the element in 1989.

4.1.1 Properties of Selenium

Selenium is classified in the group VIA in the periodic table of elements. It has both metallic and nonmetallic properties, resulting in its unique chemistry and biochemistry (Reviewed by Sunde, 2000). Selenium's 34 electrons are distributed with 18 in the argon shell, 10 3d electrons, and 6 electrons in the 4s and 4p orbitals. The 4s and 4p electrons, when lost, give rise to the common +6 and +4 oxidation states, whereas the addition of 2 electrons to the 4p orbitals completes the octet to yield the -2 oxidation state. The atomic weight of the naturally abundant isotope of selenium is 78.96.

4.1.2 Selenoproteins

Physiologically, selenium functions in the form of selenium-dependent enzymes, incorporating selenium as selenocysteine in the catalytic centers of the proteins. Selenocysteine, a seleno-analog of cysteine, is cotranslationally incorporated into selenoproteins at UGA codons, but only when specific stem-loop structures are present in the 3' untranslated region of the mRNAs. Conserved primary sequence and secondary structural features in these elements are required for selenocysteine incorporation. When these structures, termed Sec insertion sequence (SECIS) elements, are absent or disrupted, the default function of UGA codons, termination of protein synthesis, is utilized. Thus, a deficiency in dietary selenium results in decreased levels of selenoproteins, compromising biological processes that are maintained by these proteins (Copeland, 2003).

Nearly three dozen selenoproteins have been identified, though many have roles that have not been fully elucidated (Rayman, 2000). These selenoproteins have been subdivided into

groups based on the location of selenocysteine in selenoprotein polypeptides. The first group (called glutathione peroxidase, GPX) is the most abundant and includes proteins in which selenocysteine is located in the N-terminal portion of a relatively short functional domain. These include the four GPXs, selenoproteins P, Pb, W, W2, T T2 and BthD (from *Drosophila*). The second group of eukaryotic selenoproteins is characterized by the presence of selenocysteine in C-terminal sequences. These include the three thioredoxin reductases and the G-rich protein from *Drosophila*. Other eukaryotic selenoproteins are currently placed in the third group that consists of the three deiodinase isozymes, selenoproteins R and N, the 15 kDa selenoprotein and selenophosphate synthetase. The four GPXs are located in different parts of tissues and all detoxify to various degrees hydrogen peroxide and fatty acid derived hydroperoxides and thus are considered antioxidant selenoenzymes. The three deiodinases convert thyroxine to triiodothyronine, thus regulating thyroid hormone metabolism. The thioredoxin reductases reduce intramolecular disulfide bonds and, among other reactions, regenerate vitamin C from its oxidized state. These reductases can also affect the redox regulation of a variety of factors, including ribonucleotide reductase, the glucocorticoid receptor and the transcription factors (Arner and Holmgren, 2000). Selenophosphate synthetase synthesizes selenophosphate, which is a precursor for the synthesis of selenocysteine (Copeland, 2003). The functions of the other selenoproteins have not been definitely identified.

4.2 Metabolic Aspects of Selenium

4.2.1 Sources and Bioavailability of Selenium

Selenium is found in organic (selenocysteine and selenomethionine) and inorganic (selenite and selenate) forms. The organic form is found predominantly in grains, fish, meat, poultry, eggs and dairy products, and enters the food chain via plant consumption. Some nuts, in

particular Brazil nuts and walnuts, are also very good sources of selenium (Food and Nutrition, Institute of Medicine, 2000). Animals that eat grains or plants that were grown in selenium-rich soil have higher levels of selenium in their muscle.

4.2.2 Selenium homeostasis: absorption, transport, tissue distribution and excretion

The metabolism of selenium depends on the chemical form of ingested selenium. Absorbed selenium will go predominately into one of three metabolic pools. Some selenium will be used to produce selenoproteins, proteins that require selenium for catalytic activity. Selenoproteins incorporate selenium as selenocysteine (SeCys) into the polypeptide chain by using UGA as the encoding codon (Burk and Hill, 1993). Other forms of selenium will go primarily into selenium-containing proteins, proteins that do not require selenium for catalytic activity and apparently incorporate selenium randomly by substituting selenomethionine (SeMet) for methionine. Finally, all forms of selenium can go into a pool that can be methylated and excreted through the urine and lungs. Selenium consumed as a salt (selenite or selenate) will go into selenoproteins or be excreted (Whanger, 1986). Much plant material contains selenium in the form of SeMet, and SeMet will be incorporated into selenium -containing proteins to a greater extent than will selenium salts (Beilstein and Whanger, 1986; Butler et al., 1990), although it also can be degraded to selenide and then be incorporated into selenoproteins. Other forms of selenium, especially methylated derivatives, apparently go primarily into the excretion pathway (Ip et al., 1991).

4.3 Health Conditions Associated with Selenium Toxicity

4.3.1 Biomarker of selenium intake and status

The optimal intakes of selenium are determined by the intake necessary to maximize the activity of the antioxidant enzyme glutathione peroxidase in plasma, which occurs at a plasma

selenium concentration range of 89-114 μ g per liter (Reviewed by Rayman, 2000). The recommended daily allowance (RDA) of selenium for different age groups is listed in Table 4.1 (Food and Nutrition Board, Institute of Medicine, 2000).

4.3.2 Pathological consequences of selenium toxicity

At doses in excess of 900 μ g per day, selenium produces a toxic syndrome consisting of dermatitis, loose hair, diseased nails, and peripheral neuropathy (Yang et al., 1983). Other symptoms may include gastrointestinal disturbances, a garlic breath odor, fatigue, and irritability. Acute and fatal toxicities have occurred with accidental or suicidal ingestion of gram quantities of selenium.

4.4 Health Conditions Related to Selenium Deficiency

Selenium deficiency is most commonly seen in parts of China where the selenium content of the soil is very low (See Section 4.1). In areas where deficiency is found, dietary intake is less than 19 μ g per day for men and less than 13 μ g per day for women (Levander, 1991). This intake is significantly lower than the current RDA for selenium of 55 μ g per day. Selenium deficiency has been seen in people who rely on total parenteral nutrition (TPN) as their sole source of nutrition (Abrams et al., 1992). Severe gastrointestinal disorders, such as Crohn's disease, or in individuals who have had over half of their small intestine surgically removed, may decrease the absorption of selenium, resulting in selenium depletion or deficiency (Rannem et al., 1998).

Research indicates that selenium deficiency does not usually cause illness by itself. However, it can make the body more susceptible to illnesses caused by other nutritional, biochemical or infectious stresses (Institute of Medicine, Food and Nutrition Board 2000).

4.4.1 Selenium and Cellular Immunity

It is well established that adequate levels of Se are necessary for optimal function of the immune system and for cellular immunity in particular, to function properly. In two small studies, healthy (Roy et al., 1994; Kiremidjian-Schumacher et al., 1994) and immunosuppressed individuals (Kiremidjian-Schumacher et al., 2000) supplemented with 200 µg/day of selenium as sodium selenite for 8 weeks showed an enhanced immune cell response to foreign antigens compared with those taking a placebo. Se supplementation in culture increases the cytotoxicity of killer T cells as well as the proliferation of T cells in response to mitogens and antigens (Kiremidjian-Schumacher et al., 1992), whereas Se deficiency has the opposite effect. Se has also been shown to intensify the action of interleukin 2 (IL-2) by upregulating the IL-2 receptor (Roy et al. 1993).

4.4.2 Selenium and Viral Infection

Selenium deficiency appears to enhance the virulence or progression of some viral infections. The increased oxidative stress resulting from selenium deficiency may induce mutations or changes in the expression of some viral genes. The significance of the role of Se in disease progression has been documented in viruses including hepatitis b, coxsackievirus/Keshan disease, viral hemorrhagic fever, and the mouse mammary tumor virus. In these cases, Se deficiency combined with a viral cofactor causes the disease to progress (Reviewed by Taylor et al., 1996; 2000).

Keshan disease is a classical Se deficient disease whose viral cofactor is the coxsackievirus (See Section 4.1). The common form of this virus is generally benign, causing symptoms no more serious than a common cold or sore throat. The coxsackievirus only becomes virulent when Se is deficient in the body, triggering a mutation in the virus. Dr. Melinda Beck

(1995) showed that even a non-virulent strain of coxsackievirus becomes virulent in Se-deficient mice. Later, by comparing the genetic structure of the benign parent coxsackievirus to that of its virulent descendants, Beck et al. (1995) identified six specific changes in the genetic structure of the virulent coxsackievirus strain. The coxsackievirus infection is made worse because selenium deficiency weakens the host's immunity, preventing the virus from being effectively challenged by T-cell lymphocytes or antibodies (Beck et al., 1995). As a result, the mutated virus can reproduce faster than it would in a relatively healthy person. In addition, the lack of selenium prevents the quenching of mutation-causing free radicals, so when the virus reproduces, it also mutates at a faster rate. Although it is unclear whether one or all of these genetic changes triggered the more aggressive virus, the genetic evidence provides the scientific proof needed to link a host's selenium deficiency with a more dangerous form of the coxsackievirus.

In addition to Keshan disease, Kashin-Beck disease is associated with poor selenium status in areas of northern China, North Korea, and eastern Siberia. Kashin-Beck disease affects prepubescent children, and is characterized by the degeneration of the articular cartilage between joints (osteoarthritis). There is little evidence that improving selenium nutritional status prevents Kashin-Beck disease. A number of other causative factors have been suggested for Kashin-Beck disease, including fungal toxins in grain, iodine deficiency, and contaminated drinking water (Burk and Levander, 1999).

4.4.3 Oxidative Stress

Se plays an important role in both immunologic function and antioxidant defense. Antioxidant defense systems are needed to protect the body against reactive oxygen species (ROS), unavoidable byproducts of oxygen metabolism. Oxidative damage caused by free radicals results in many detrimental conditions including chronic diseases. Se-containing

antioxidants work to break down peroxides and reverse lipid peroxidation (Tapiero et al., 2003)

4.5 Selenium and HIV

Selenium seems to be a crucial nutrient for HIV-infected individuals. The mechanisms by which selenium influences the progression of these diseases remains unknown, but the potential significance of the role of selenium in AIDS progression may be similar to that of the other viruses.

4.5.1 Acquired Immunodeficiency Syndrome (AIDS)/ Human Immunodeficiency Virus (HIV)

At the end of 2002, the Pan American Health Organization (PAHO) reported that 42 million people are estimated to be living with HIV/AIDS. Few crises have affected human health and threatened national, social and economic progress in quite the way that HIV/AIDS has. With no cure for this disease, medical nutrition therapy is a large part of treatment. Providing nutritional care and support for people living with HIV/AIDS is an important part of caring at all stages of the disease.

Primary infection with HIV is the underlying cause of AIDS. HIV invades the genetic core of the CD4+T or T-helper lymphocyte cells. Although HIV needs CD4+ cells to be activated before it can replicate, CD4+ cells are the principal agents involved in protection against infection. Distinct viral compartments that have been identified are blood, semen, vaginal secretions, the lymph system, and the central nervous system. HIV infection causes a progressive depletion of CD4+ cells, which eventually leads to immunodeficiency, constitutional disease, neurologic complications, opportunistic infections, and neoplasms (Webb and Norton, 2004).

HIV-1 infection is characterized by an initial acute viremic stage followed by years of clinical latency prior to disease escalation and the manifestation of acquired immunodeficiency

syndrome (AIDS). The progression time from infection with HIV to the development of AIDS is extremely variable. The fact that a minority of infected persons remain AIDS-free without overt signs of disease progression many years after infection combined with the findings that Se status predicts HIV outcome indicates that nutritional status is one of various factors that may be involved in these cases (Taylor et al., 2000).

4.5.2 Evidence of a link between selenium and HIV/AIDS

Over the span of the last decade, the progressive decline of serum selenium levels paralleling the loss of CD4⁺T cells has been widely documented in HIV-1 infections, with results published in no less than 20 papers (Look et al., 1997). These findings have been discovered in both symptomatic and asymptomatic patients, indicating that malnutrition or nutrient malabsorption cannot entirely explain this decline (Reviewed by Taylor, 1997).

Evidence increasingly suggests that a selenium deficit is not only a correlate of disease progression, but is actually a powerful predictor of mortality vs. survival in AIDS. It was recently reported that the risk of dying from AIDS within a 3.5 year period in HIV⁺ patients with reduced selenium levels is 20-fold higher than that of patients with normal selenium levels (Baum et al., 1997). Other studies suggest that selenium may play a role in preventing the transmission of HIV. Baeten et al. (2001) reported that women who are HIV⁺ and are selenium deficient have an almost threefold greater likelihood of shedding genital mucosal HIV-1 DNA.

Results from numerous cross-sectional studies reported that AIDS patients exhibit clinical selenium deficiency (defined as < 85 micrograms (µg) Se/L) or plasma selenium concentrations lower than non-infected controls (Reviewed in Rayman, 2000). In HIV infection, decreased serum or erythrocyte selenium levels have been associated with increased progression to AIDS, greater incidence of opportunistic infections, and decreased survival. Selenium

deficiency appears to be related to poor prognosis and to specific clinical characteristics of HIV disease. Additionally, it has been demonstrated that selenium deficiency, independent of CD4+T-cell counts and antiretroviral treatment, is a significant predictor of HIV-related mortality.

In a recent one-year, double-blind, placebo-controlled trial, a high-selenium nutritional supplement selenium (400 µg) showed a reduction of 74% in mortality for people with T4's fewer than 100 ($p = .03$) and a reduction of 63% for people with T4's fewer than 200 ($p = .05$) (Jiamton, 2003). The reduction in deaths was not caused by improvements in T4 counts or viral load. In fact, there was no difference in changes in T4's or viral loads between the supplement and the placebo groups. Neither was there any difference in hospital admission rates between the two groups. The researchers suggested that "a novel mechanism independent of CD4T-lymphocyte numbers" accounted for the improvement in survival.

Burbano et al. (2002) evaluated the impact of selenium chemoprevention (200 µg/day) on hospitalizations in 186 HIV-positive men and women. This randomized, double-blind, placebo-controlled trial (1998-2000) indicated that there was a marked decrease in total hospital admission rates ($RR = 0.30$; $p = .002$) and percent of hospitalizations due to infection/100 patients for those receiving selenium. In another study, Sacher (1994), of Frankfurt, Germany, reported that selenium-supplemented AIDS patients gain weight, have a general feeling of well-being, and sometimes benefit from increases in protective CD4 T-cells.

Campa et al. (1999) reported that in pediatric HIV-infection, low plasma level of selenium is an independent predictor of mortality, and appears to be associated with faster disease progression. They found that only CD4 cell count below 200, and low levels of plasma selenium were significantly and independently related to mortality.

4.6 Viral Selenoprotein Theory

The genomes of both bacteria and eukaryotic organisms are known to encode selenoproteins, using the UGA codon for seleno-cysteine (SeC), and a complex cotranslational mechanism for SeC incorporation into polypeptide chains, involving RNA stem-loop structures (Taylor et al., 1997). However, the possibility that some viruses might also encode selenoproteins remained unexplored until recently.

It is hypothesized that during HIV infection, several viral selenoproteins are expressed by ribosomal frameshifting and/or suppression of termination codons, and synthesis of these viral selenoproteins deplete the selenium pool of the host (Taylor et al., 1994).

4.6.1 HIV Encoded Selenoproteins

Based on an analysis of the genomic structure of the human immunodeficiency virus (HIV-1), several regions overlapping known HIV genes with the potential to encode selenoproteins were identified (Taylor, 1994). While studying the HIV genome, Taylor et al. discovered several RNA pseudoknots in the protease and polymerase coding regions. These pseudoknots led to the discovering the novel genes, since pseudoknots are known to be involved in frameshifting, a process required for the expression of these hypothetical proteins.

4.6.2 An HIV-Encoded Selenium-Dependent GPx Gene

Several studies have suggested that HIV-infected cells have altered antioxidant defenses. The role of the cytosolic form of glutathione peroxidase is of particular interest in influencing the course of an HIV-1 infection. Glutathione peroxidase (GPx) is a key enzyme for the removal of hydrogen peroxide and organic peroxides. By removing these peroxides, GPx prevents the accumulation of highly toxic hydroxy radicals. The activity of glutathione peroxidase is dependent on the constant flux of its co-substrate, GSH, and on adequate levels of selenium. It is

therefore, not surprising that in cell culture studies, selenium, as a component of the selenoprotein GPx, has been consistently found to be an inhibitor of NF- κ B activation, presumably due to the reduction of peroxide levels by GPx (Reviewed by Taylor et al., 2000).

Taylor et al. have shown that HIV can encode a homologue of GPx by a ribosomal frameshift from the *env* coding region. They have cloned this gene and shown it to have functional GPx activity when expressed as a selenoprotein in human cells (Zhao et al., 2000). Furthermore, the cysteine homologue (selenocysteine to cysteine mutant) of the viral GPx has been expressed in bacteria and isolated: the purified 9-kd protein has significant GPx activity, although that activity is low, as would be expected for a cysteine mutant of a selenium-dependent GPx (Taylor, 2000).

4.6.3 The 3' Terminal of HIV-1 *nef* is a UGA Codon in a Motif Identical to the Thioredoxin

Reductase C-Terminal Redox Center

The Nef protein of human immunodeficiency virus type 1 (HIV-1) stimulates viral infectivity. Aiken and Trono (1995) found that viruses containing disrupted *nef* genes were 4 to 40 times less infectious than wild-type HIV-1 in a single-round infection. The Nef effect is dependent on the association of this protein with the plasma membrane and is determined at the stage of the virus particle formation.

The 3'-terminal UGA codon of *nef* is highly conserved in the known group M HIV-1 isolates (in 99% of known sequences); which is a unique feature of HIV-1 compared with HIV-2 and SIV *nef* genes. HIV-2 and SIV *nef* genes contain UAA and UAG stop codons at the 3' end in various subtypes, with no particular bias. Conservation of this 3'-terminal UGA codon is a striking difference between the more pathogenic HIV-1 versus that of other primate viruses (Taylor et al., 2000).

Preliminary studies suggest that read-through suppression of the *nef* 3' UGA codon can occur in vivo, and that in vitro this effect is selenium dependent (Taylor et al, 1997). Another relevant study confirmed that thioredoxin reductase (TrxR), whose major activity is to reduce activation of NF-kB, is a selenoprotein. The *nef* terminal contains a conserved cysteine residue immediately preceding the UGA codon, making it identical to that of the redox center of TrxR (Gorlatov et al., 1998). In addition, a sequence downstream of the UGA codon is quite similar to the sequence of a second disulfide redox center located near the N-terminal of TrxR, a cysteine-selenocysteine pair (Reviewed by Taylor, 2000). The working theory is that HIV-1 is using a viral selenoprotein for NF-kB activation, whereas the HIV-1 vGPx (a late gene) would be expected to deactivate NF-kB by decreasing oxidant tone.

4.6.4 HIV-1-Encoded NF-kB Homologue (*pro-fs*)

Taylor et al. (1994) discovered the existence of a potential HIV-1 selenoprotein module potentially expressed by a -1 frameshift from the protease coding region, which was later named *pro-fs*. This selenoprotein was initially compared to the papillomavirus E2 DNA binding protein, which has a conserved cysteine residue that aligns with the highly conserved UGA codon of *pro-fs*. Members of the NF-kB family of transcription factors also contain an essential cysteine residue in their DNA binding domain; in fact, this cysteine amino acid is what TrxR maintains in a reduced state in order for NF-kB to bind to DNA (Reviewed by Taylor et al., 2000). Subsequent studies have demonstrated that *pro-fs* is a potent activator of HIV-1 replication in vitro. The activating effect appears to be mediated exclusively via the NF-kB recognition sites in the long terminal repeat (LTR).

4.7 Research Project

One predicted selenium-incorporating site found by Taylor et al. (1994), later termed *pro-fs* due to expression by a -1 frameshift from the protease coding region, has been found to be a potent activator of HIV-1 replication in transfected Madin-Darby Canine Kidney (MDCK) cells (Taylor et al., unpublished). The Madin-Darby Canine Kidney (MDCK) cell line is an established line of canine kidney cells that has been used in several laboratories to study virus-host cell relationships. When grown in tissue culture, MDCK cells have the morphological properties of distal tubular epithelial cells (Rindler, 1979). Two-dimensional gel electrophoresis (2DGE) was used to identify alterations in cellular protein expression, due to transfection with the *pro-fs* construct, in Madin-Darby Canine Kidney (MDCK) cells. Elucidation of the importance of novel viral selenoproteins may improve our understanding of HIV.

Table 5.1: Dietary Reference Intake for Selenium

Nutrient	Life Stage Group	RDA/AI*	UL ^a
Selenium	Infants	(µg/d)	(µg/d)
	0- 6 mo	15*	45
	7-12 mo	20*	60
	Children		
	1- 3 y	20	90
	4- 8 y	30	150
	Males		
	9-13 y	40	280
	14- 18 y	55	400
	19- 30 y	55	400
	31-50 y	55	400
	50-70 y	55	400
	> 70 y	55	400
	Females		
	9-13 y	40	280
	14- 18 y	55	400
	19- 30 y	55	400
	31-50 y	55	400
	50-70 y	55	400
> 70 y	55	400	
Pregnancy			
= 18 y	60	400	
19-30y	60	400	
31-50 y	60	400	
Lactation			
= 18 y	70	400	
19-30y	70	400	
31- 50 y	70	400	

NOTE: The table is adapted from the DRI reports, see www.nap.edu. It represents Recommended Dietary Allowances (RDAs) in **bold type**, Adequate Intakes (AIs) in ordinary type followed by an asterisk (*), and Tolerable Upper Intake Levels (ULs)^a. RDAs and AIs may both be used as goals for individual intake. RDAs are set to meet the needs of almost all (97 to 98 percent) individuals in a group. For healthy breastfed infants, the AI is the mean intake. The AI for other life stage and gender groups is believed to cover the needs of all individuals in the group, but lack of data prevent being able to specify with confidence the percentage of individuals covered by this intake.

^aUL = The maximum level of daily nutrient intake that is likely to pose no risk of adverse effects. Unless otherwise specified, the UL represents total intake from food, water, and supplements.

CHAPTER 6
PROTEIN EXPRESSION OF MDCK CELLS
TRANSFECTED WITH *PRO-FS*, A POTENTIAL SELENOPROTEIN MODULE²

² Erica Renee Berelc, Michael Frank Mouat, Ting Li, Ethan Will Taylor, Arthur Grider. To be submitted to *Proteome Science*.

Abstract:

It is hypothesized that during HIV infection, several viral selenoproteins are expressed by ribosomal frameshifting and/or suppression of termination codons, and synthesis of these viral selenoproteins deplete the selenium pool of the host. One predicted selenium-incorporating site, termed *pro-fs* due to expression by a -1 frameshift from the protease coding region, has been found to be a potent activator of HIV-1 replication. The activating effect appears to be mediated exclusively via the NF- κ B recognition sites in the long terminal repeats (LTR). In this study, two-dimensional gel electrophoresis (2DGE) of Madin-Darby Canine Kidney (MDCK) cells were used to identify alterations in cellular protein expression due to the transfection with the *pro-fs* construct. After staining with Sypro Ruby, more than 400 proteins spots were visualized, with 42 protein spots identified as being differentially expressed. Differentially expressed proteins were excised, digested with trypsin, and subjected to MALDI-TOF mass spectrometry. Proteins that were upregulated due to transfection with the *pro-fs* construct included cytoskeletal proteins (tropomyosin, keratin, and actin), histone 2B, ornithine aminotransferase precursor, and heat shock proteins. Heat shock proteins play a role in signal transduction by forming complexes with nuclear receptors and protein kinases, such as Raf and p21ras. Both p21ras and its downstream Raf-1 are activated in human immunodeficiency virus-infected monocytes, and participate in the activation of NF- κ B.

INDEX: selenium, selenoproteins, HIV, Madin-Darby Canine Kidney, proteomics, two-dimensional gel electrophoresis, pro-fs, mass spectrometry

Background

Selenium (Se) is an essential trace element that is necessary for optimal function of the

immune system, playing an important role in both immunologic function and antioxidant defense. Selenium has been found to be a chemoprotective agent against cancer and several viral diseases including hepatitis b, coxsackievirus/Keshan disease, viral hemorrhagic fever, and the mouse mammary tumor virus. A progressive decline in plasma or serum selenium levels, paralleling the loss of CD4+T cells, has been widely documented in HIV-1 infections. The mechanisms by which selenium influences the progression of these diseases remains unknown, but the potential significance of the role of selenium in AIDS progression may be similar to that of the other viruses.

Based on an analysis of the genomic structure of the human immunodeficiency virus (HIV-1), several regions overlapping known HIV genes with the potential to encode selenoproteins were identified (Taylor, 1994). One of these potential HIV-1 selenoprotein modules is expressed by a -1 frameshift from the protease coding region. This selenoprotein, termed *pro-fs*, was initially compared to the papillomavirus E2 DNA binding protein, which has a conserved cysteine residue that aligns with the highly conserved UGA codon of *pro-fs*. Members of the NF- κ B family of transcription factors also contain an essential cysteine residue in their DNA binding domain; in fact, this cysteine amino acid is what Thioredoxin (Trx) maintains in a reduced state in order for NF- κ B to bind to DNA (Reviewed by Taylor et al., 2000). In transfected Madin-Darby Canine Kidney (MDCK) cells, *pro-fs* has been found to be a potent activator of HIV-1 replication. The activating effect appears to be mediated exclusively via the NF- κ B recognition sites in the long terminal repeat (LTR).

The MDCK cell line is an established line of canine kidney cells that has been used in several laboratories to study virus-host cell relationships. When grown in tissue culture, MDCK cells have the morphological properties of distal tubular epithelial cells (Rindler, 1979). Two-

dimensional gel electrophoresis (2DGE) was used to identify alterations in cellular protein expression, due to transfection with the *pro-fs* construct, in MDCK cells. Elucidation of the importance of novel viral selenoproteins may improve our understanding of HIV.

Results

MDCK cell lysates (0.1 mg) were electrophoretically separated by isoelectric focusing in the first dimension and by SDS-12% polyacrylamide gel electrophoresis in the second dimension. Transfection with the *pro-fs* construct was accomplished using Lipofectamine™ 2000 and manufacturer's procedure (Invitrogen, 2002-2003). Proteins were visualized by staining with Sypro Ruby. Of the more than 400 proteins visible by Sypro Ruby staining, 42 spots were excised from the gel and subjected to in-gel tryptic digestion. The resulting peptides were analyzed and identified by MALDI-TOF mass spectrometry.

A typical 2DGE gel representing normal MDCK cells and stained with Sypro Ruby is shown in figure 6.1. A typical DGE gel representing MDCK cells that were transfected with the *pro-fs* construct, and stained with Sypro Ruby is shown in figure 6.2. Identified proteins are summarized in Tables 6.1 and 6.2. We found that transfection with the *pro-fs* construct resulted in the differential expression of 42 proteins.

Discussion

In the present study, we used the MDCK cell line to identify the effect of transfection with the *pro-fs* construct on protein expression. The results of 2DGE in conjunction with MS indicated the differential expression of numerous proteins (Tables 6.1 and 6.2). Proteins that were upregulated due to transfection with the *pro-fs* construct included cytoskeletal proteins (tropomyosin, keratin, and actin), heat shock proteins, ornithine aminotransferase precursor, and histone 2B.

The cytoskeleton regulates cell shape, transport, motility, and integrity. It consists of 3 protein filament systems with a large number of associated proteins that regulate each system. Microfilaments consist of actin and tropomyosin (Spots 30, 31) in association with a large number of other proteins. In mammalian nonmuscle cells, 2 classes of actin are recognized on isoelectric focusing gels: beta and gamma. These 2 isoforms differ by 4 amino acid substitutions at the conserved NH₂-end of the molecule. They are coexpressed in nonmuscle cells. Gamma-actin (Spot 38) and beta-actin (Spot 40) were upregulated in our transfected cell line. Keratin, type 1 cytoskeletal 18 (Spot 39) and keratin, type 2 cytoskeletal (Spot 36) were also upregulated due to transfection. These two types of cytoskeletal and microfibrillar keratin: I (acidic; 40-55 kDa) [K9 to K20] and II (neutral to basic; 56-70 kDa) [K1 to K8] are normally associated with each other.

Heat shock proteins (Spots 7, 9) are a family of cellular proteins characterized by their up-regulation in response to stress and the presence of a weak ATPase activity (Richter et al., 2001). They were initially described as chaperones that facilitate the folding of other proteins. However, recent studies (Aligue et al., 1994; Cutforth and Rubin, 1994; Nathan and Lindquist, 1995; Pratt and Toft, 1997) indicate that heat shock proteins also play a role in cytoprotection, anti-apoptosis, and signal transduction by forming complexes with nuclear receptors and protein kinases, such as Raf, Src, p21 *ras*, p53, and Rb.

The mitochondrial matrix protein p1 precursor (Spot 33), also known as the 60 kDa heat shock protein, is implicated in mitochondrial protein import and macromolecular assembly. This protein may facilitate the correct folding of imported proteins; as well as prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix. This protein also interacts with p21Ras.

Transcriptional control of human immunodeficiency virus type 1 (HIV-1) in T lymphocytes involves a complex interaction between cellular and viral regulatory proteins and their target sequences within the long terminal repeat (LTR) (Gaynor, R. 1992). The NF- κ B-binding motif in the HIV LTR is a Raf-responsive element (Finco and Baldwin, 1993). Both p21ras and its downstream Raf-1 are activated in human immunodeficiency virus-infected monocytes, and participate in the activation of NF-kappa B (Folguiera et al, 1996).

Histone (Spot 41) upregulation is indicative of increased cell proliferation. *Pro-fs* has been found to be a potent activator of HIV-1 replication. The upregulation of histone 2B, may suggest that host cell proliferation is increased as well. Ornithine aminotransferase (Spot 37) was also found to be upregulated due to *pro-fs* transfection, though the reason is unknown at this time.

Mass spectrometry (MS) data were acquired on the 4700 Proteomics Analyzer (Applied Biosystems) using standard acquisition methods. Mass lists were submitted to NCBI Inr and SwissProt using Mascot (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) (Mammals). Identifications were cross-examined using mass accuracy, molecular weight, and pI. Though mass list were not submitted to a canine database, results were similar.

Pro-fs has a computed pI of 11 and mass of 7.2 or 8.3 kDa, depending on whether one or both in-frame UGA codons are decoded as selenocysteine. Such a basic protein could not be visualized using our 2DGE gel protocol. Therefore, future samples will be run in the isoelectric focusing dimension using non-equilibrium pH gel electrophoresis (NEPHGE). This technique increases the separation of proteins with basic isoelectric points.

Materials and Methods

Cell culture media and chemicals were obtained from Sigma Chemical Co., Summit Biotechnology, Invitrogen, and/or Gibco Laboratories.

Cell Culture

The Madin Darby Canine Kidney cell line was maintained in DMEM tissue culture medium (Sigma Chemical Company, St. Louis, Missouri) supplemented with 10% fetal calf serum (Summit Biotechnology, Ft Collins, Colorado) and at 37° in 5.0% CO₂. Transfection with the *pro-fs* construct was accomplished using Lipofectamine™ 2000 and manufacturer's procedure (Invitrogen, 2002-2003).

Preparation of total cell lysate

The wells were rinsed three times (5 minutes each time) with 4 mL ice-cold wash buffer (2.7 mM KCl, 1.5 mM KH₂PO₄, 136 mM NaCl, 8.1 mM Na₂HPO₄·7 H₂O, 18 meg Ohm water). After removal of the final volume of wash buffer, 30 µL of boiling sample buffer 1 (0.3% SDS, 200 mM DTT, 28 mM Tris HCl, 22 mM Tris base, 18 meg Ohm water) was added to each well, the cells scraped together into the buffer and the cell lysate transferred into 1.5-mL microfuge tubes. The cell lysate was heated for five minutes at 100°C, chilled on ice for 5 minutes, and 9 µL of sample buffer 2 (24 mM Tris base stock, 476 mM Tris HCl stock, 50 mM MgCl₂ stock, 1.0 mg/mL DNase 1, 0.25 mg/mL RNase A (10 mg/mL) was added. After incubation on ice for 8 minutes, the cellular proteins were precipitated by the addition of acetone to 80% v/v and incubation on ice for twenty minutes. The microfuge tubes were then centrifuged at 12,000 X g for ten minutes at 4°C, the supernatant discarded and the pellet dried at room temperature for five minutes. The pellet was resuspended in 240 µL of IPG Sample Buffer (7M Urea, 2M Thiourea, 4% CHAPS, 2% Pharmalyte (3-10), 1% DTT), and an aliquot was removed for assaying the

protein concentration. The remainder was stored at -80°C . The protein concentration was determined using the Bradford method (Bradford 1976, Bio-Rad, Hercules, CA).

2DPAGE- first dimension isoelectric focusing (IEF)

The proteins from MDCK normal and MDCK cells transfected with *pro-fs* were separated by two-dimensional gel electrophoresis. Samples were mixed with rehydration buffer (6M Urea, 2M Thiourea, 2% CHAPS, 0.5% Pharmalyte (3-10), 0.4% DTT) to a total volume of 300 μL at a concentration of 2 mg/mL and loaded onto a precast immobilized pH gradient (IPG) gel strip (Bio-Rad). They were then overlaid with mineral oil and allowed to rehydrate under passive condition for 12-16 hours at 20°C . First dimensional IEF was performed for 60,000 volt-hours.

2DPAGE- second dimension

After IEF, the IPG strips were equilibrated with 6 M urea, 30% glycerol, 2% SDS, 1.55 M Tris, 2 mM TBP, for 25 min at room temperature, then loaded onto large format (22 cm X 22 cm X 1 mm) 12% acrylamide slab gels. The gels were run at 20 W/gel at 4°C until the dye front reached within 1 cm from the bottom of the gel. The cathode buffer contained 50 mmol/L Tris base, 384 mmol/L glycine and 6.9 mmol/L sodium dodecyl sulfate. The anode buffer contained 25 mmol/L Tris base, 192 mmol/L glycine and 3.5 mmol/L sodium dodecyl sulfate.

Staining

After running the second dimension, the gels were fixed in 10% ethanol/ 7% acetic acid for 30 minutes. The gels were allowed to incubate overnight in diluted (2X) Sypro Ruby staining solution with gentle agitation. The gels were destained in 10% ethanol/ 7% acetic acid for 60 minutes. After washing with H_2O , the gels were visualized using a blue-light transilluminator.

Analysis

The spots that were differentially expressed or up regulated were excised from six gels. The gel pieces were sent to the Proteomics Resource Facility (University of Georgia, Athens, GA) for in-gel digestion and amino acid sequencing. Two mm gel plugs were picked, washed, digested with trypsin; the resulting peptides were extracted and spotted using the Spot Handling Workstation (Amersham Biosciences). Briefly, plugs were washed twice with 50 mM ammonium bicarbonate/50% methanol for 20 minutes at room temperature. Plugs were washed with 75% acetonitrile for 20 minutes at room temperature and dried at 40°C for 10 minutes. Plugs were then incubated with 140 ng sequencing grade trypsin (Promega) at 37°C for 1 hour. Peptides were extracted twice with 50% acetonitrile/0.1% TFA for 20 minutes at room temperature. Approximately 25% of the resulting peptides were spotted with partially saturated *o*-cyano-4-hydroxy-cinnamic acid (Sigma). Mass spectrometry (MS) data were acquired on the 4700 Proteomics Analyzer (Applied Biosystems) using standard acquisition methods. MS spectra were calibrated using two trypsin autolysis peaks (1045.45 and 2211.096 m/z). Mass lists were submitted to NCBIInr and SwissProt using Mascot (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html) (Mammals). Identifications were cross-examined using mass accuracy, molecular weight, and pI.

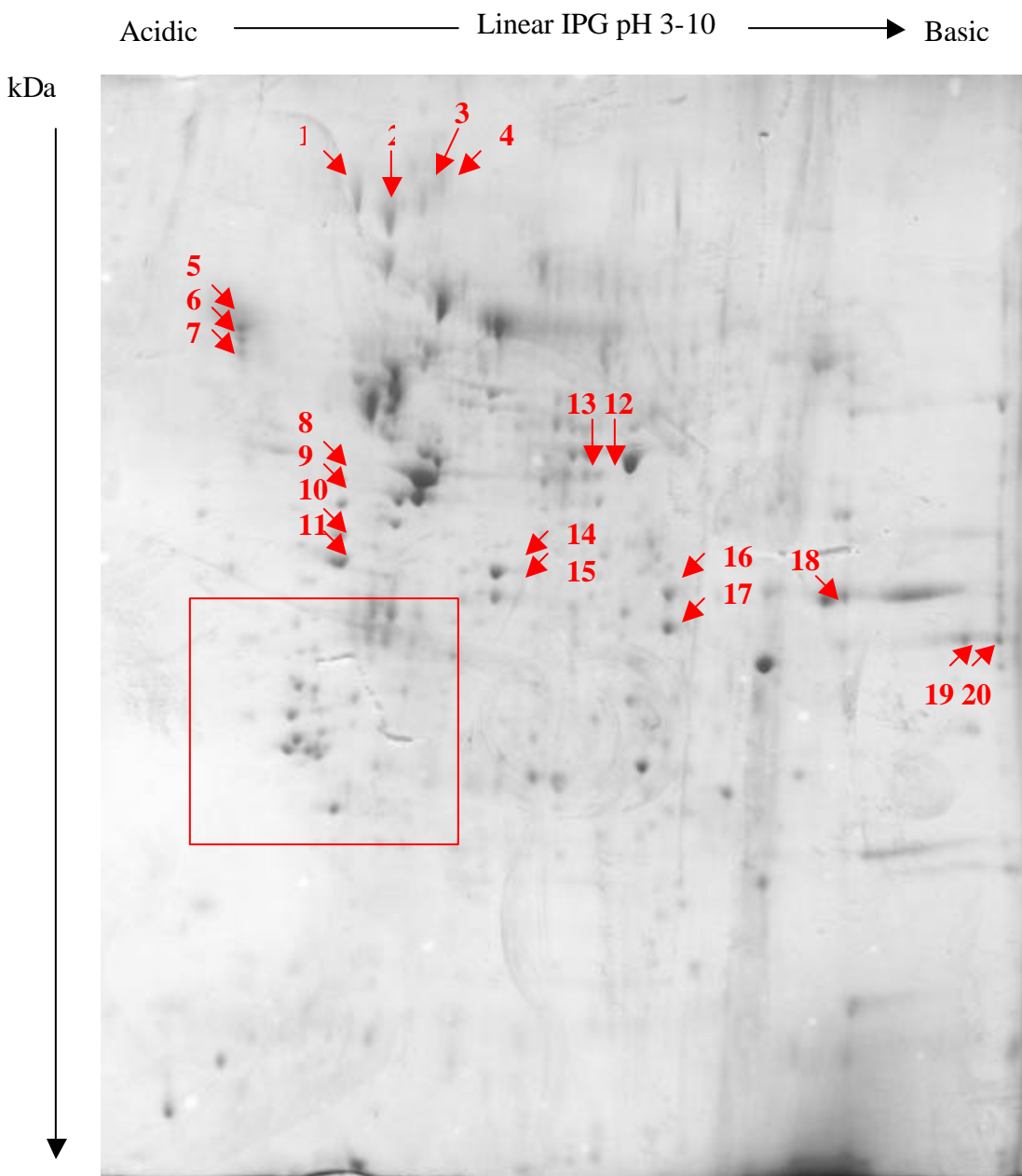


Figure 6.1: 2D image of proteins from MDCK cells that were found to be down-regulated in response to transfection with the *pro-fs* construct. Protein extracts were separated on pH 3-10 linear IPG strips. This was followed by electrophoresis on 12% SDS-polyacrylamide gels, as stated in Materials and Methods. The gel was stained with Sypro Ruby. Differential protein expression between dietary groups was analyzed by Phoretix software.

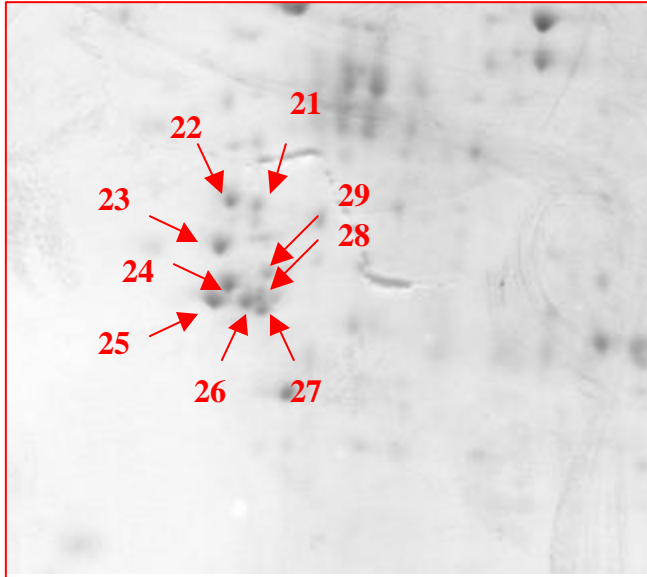


Figure 6.1. (continued): Magnified area from Figure 6.1 on previous page.

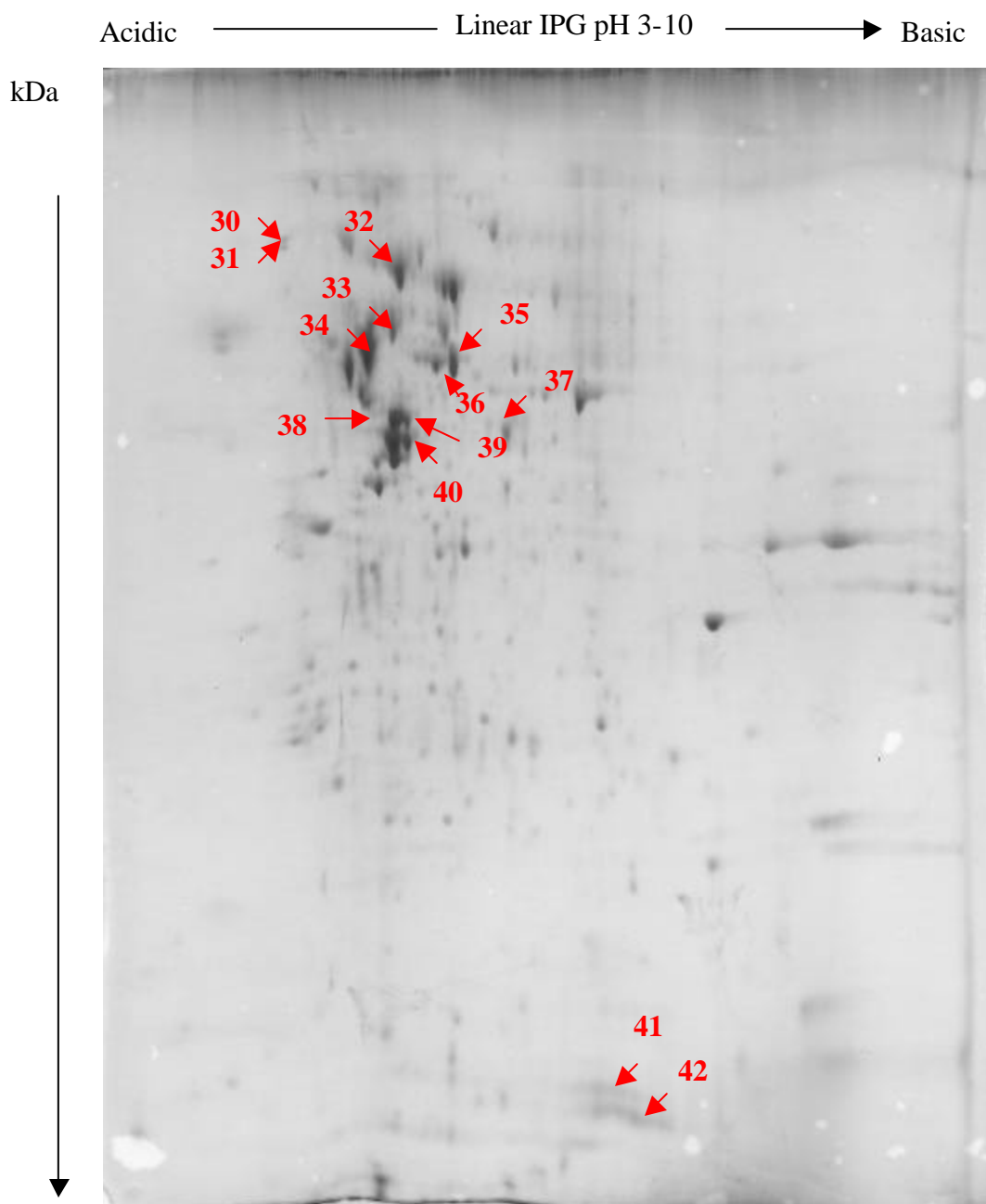


Figure 6.2: 2D image of proteins from MDCK cells found to be upregulated due to transfection with the *pro-fs* construct. Protein extracts were separated on pH 3-10 linear IPG strips. This was followed by electrophoresis on 12% SDS-polyacrylamide gels, as stated in Materials and Methods. The gel was stained with Sypro Ruby. Differential protein expression between dietary groups was analyzed by Phoretix software.

Table 6.1: Overview of proteins that were down-regulated in response to transfection with the *pro-fs* construct.

Spot No ^b	Annotation and/or Homologue Proteins ^c	Accession Number ^d	MW (kDa) ^e
1	PROTEIN KINASE PPK98 (EC 2.7.1.-)	gi 7441885	92367.2
2	HEAT SHOCK PROTEIN HSP 90-ALPHA (HSP 86)	sp P07900 HS9A_	84489.6
3	TRANSITIONAL ENDOPLASMIC RETICULUM ATPASE (TER ATPASE)	sp P03974 TERA_	89232.7
4	HEAT SHOCK COGNATE 71 KD PROTEIN	sp P11142 HS7C_	70854.2
6	CALRETICULIN PRECURSOR (CRP55)	sp P15253 CRTC_	48245
9	40S RIBOSOMAL PROTEIN SA (P40)	sp P08865 RSP4_	32833.4
12	ELONGATION FACTOR TU, MITOCHONDRIAL PRECURSOR	sp P49410 EFTU_	49367
14	ANNEXIN I	sp P04083 ANX1_	38558.9
15	ANNEXIN I	sp P04083 ANX1_	38558.9
16	ANNEXIN II	sp P07355 ANX2_	38448.8
17	ANNEXIN II	sp P07355 ANX2_	38448.8
18	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (EC 1.2.1.12)	sp P46406 G3P_R	35666.2
19	HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS A2/B1	sp P22626 ROA2_	37406.7
20	L-LACTATE DEHYDROGENASE M CHAIN (EC 1.1.1.27)	sp P06151 LDHM_	36344.2
22	TROPOMYOSIN, FIBROBLAST NON-MUSCLE TYPE (TM30-PL)	sp P07226 TPM4_	28504.5
23	14-3-3 PROTEIN EPSILON	sp P42655 143E_	29155.4
24	14-3-3 PROTEIN SIGMA	sp P31947 143S_	27756.7
25	KERATIN, TYPE I CYTOSKELETAL 9	sp P35527 K1CI_	61949.8
26	14-3-3 PROTEIN ZETA/DELTA	sp P29312 143Z_	27727.7
27	14-3-3 PROTEIN BETA/ALPHA	sp P31946 143B_	27933.8
28	14-3-3 PROTEIN ZETA/DELTA	sp P29312 143Z_	27727.7
29	14-3-3 PROTEIN GAMMA	sp P29359 143G_	27989.8

^b Refers to the proteins labeled in Figures 6.1.

^c Name of identified protein.

^d SwissProt.

^e Molecular Weight.

Note: Numbers 5, 7, 8, 10, 11, 13, 21, are unidentified at this time.

Table 6.2: Overview of proteins that were upregulated in response to transfection with the *pro-fs* construct.

Spot No ^b	Annotation and/or Homologue Proteins ^c	Accession Number ^d	MW (kDa) ^e
30	TROPOMYOSIN BETA CHAIN, PLATELET	sp P02561 TPMP_	28374.4
31	TROPOMYOSIN BETA CHAIN, PLATELET	sp P02561 TPMP_	28374.4
32	HEAT SHOCK COGNATE 71 KD PROTEIN	sp P11142 HS7C_	70854.2
33	MITOCHONDRIAL MATRIX PROTEIN P1 PRECURSOR	sp P19226 P60_M	60917.4
36	KERATIN, TYPE II CYTOSKELETAL 8	sp P05786 K2C8_	42369.5
37	ORNITHINE AMINOTRANSFERASE PRECURSOR (EC 2.6.1.13)	sp P04182 OAT_R	48302
38	ACTIN, CYTOPLASMIC 2 (GAMMA-ACTIN)	sp P02571 ACTG_	41765.8
39	KERATIN, TYPE I CYTOSKELETAL 18	sp P05784 K1CR_	47344.2
40	ACTIN, CYTOPLASMIC 1 (BETA-ACTIN)	sp P02570 ACTB_	41709.7
41	HISTONE H2B.1	sp P06899 H2B1_	13475.3

^b Refers to the proteins labeled in Figures 6.2.

^c Name of identified protein.

^d SwissProt.

^e Molecular Weight.

Note: Numbers 34, 35, 42 are unidentified at this time.

CHAPTER 7

CONCLUSION

In recent years, the development of research entailing the protein complement of the genome, the proteome, has evolved significantly as a result of improved technology for two-dimensional gel electrophoresis (2DGE), 2D image analysis, and mass spectrometry for protein identification. Using these technologies, it is now possible to obtain a more holistic view of the protein complement of a cell line, tissue, or organism.

Because proteins are key structural and functional molecules, molecular characterization of proteomes is necessary for a complete understanding of biological systems (Beranova-Giorgianni, 2003). The proteomic signatures can be the markers of a certain condition of health and can be used to monitor, for example, whether one is healthy or suffering from a certain disease.

5.1 Advantages of Proteomics

It is difficult to predict genes accurately from genomic data (Eisenberg et al., 2000). After transcription from DNA to RNA, the gene transcript can be spliced in different ways prior to translation into protein. Proteins may undergo more than 200 different types of post-translational modification, including phosphorylation, glycosylation, acetylation, deamination, farnesylation, myristoylation, palmitoylation, and proteolysis (Krishna and Wold, 1993). Such modifications play a vital role in modulating the function of many proteins but are not directly coded by genes.

As a consequence, the information from a single gene can encode as many as 50 different protein species. Only through the study of proteins themselves can their characteristics and functions be elucidated.

5.2 Limitations of Proteomics

The study of proteins presents a number of unique challenges. Firstly, it is a labor-intensive and tedious procedure. Secondly, many large or hydrophobic proteins do not enter the gel during the first dimension. In addition, proteins with extreme pHs (below 3 or above 10) are not separated, but focused as vertical lines on both sides. Finally, because there is no equivalent of PCR for proteins, the analysis of low-abundance proteins remains a major challenge.

The challenge of analyzing low abundance proteins can be overcome by increasing the amount of sample loaded onto the gel; however, there may be a risk of overloading the system and reducing the resolution. In some eukaryotic cells, the amounts of the most abundant proteins can be 10^6 -fold greater than those of the low-abundance proteins. These low-copy proteins will not be observed in the analysis of crude cell lysates without some purification. Therefore, new methods must be devised for subproteome isolation.

5.3 Zinc Deficiency Affects Protein Expression in Caco-2 Cells

We investigated the effect of zinc deficiency on protein expression in Caco-2 cells using two-dimensional gel electrophoresis (2DGE) along with mass spectrometry (MS). These methods allow the qualitative and quantitative comparison of protein expression of different samples, i.e. Caco-2 cells grown in zinc adequate versus zinc deficient media, at the time of isolation. These studies, therefore, provide a more complete picture of the adaptive response that the cell or tissue mounts following that treatment.

In addition to its diverse role in many physiological systems, zinc has been shown to be an important regulator of apoptosis. Subtle changes in cellular zinc content and localization have been reported to have profound effects on cell metabolism and function. In humans, zinc deficiency negatively affects the epidermal, central nervous, immune, gastrointestinal, skeletal, and reproductive systems.

Our conclusion is that the Caco-2 model is a good cell culture model and that proteomics techniques are powerful tools, for the study of the effect of zinc deficiency on protein expression. Future studies will focus on the role zinc plays in expression of proteins associated with cell cycle regulation, especially apoptosis.

5.4 Protein Expression of MDCK Cells Transfected with pro-fs, a Potential HIV-1

Selenoprotein

Two-dimensional gel electrophoresis (2DGE) was used to identify alterations in cellular protein expression, due to transfection with the pro-fs construct, in Madin-Darby Canine Kidney (MDCK) cells. The results indicated the differential expression of numerous proteins. Proteins that were upregulated due to transfection with the pro-fs construct included cytoskeletal proteins (tropomyosin, keratin, and actin), heat shock proteins, ornithine aminotransferase precursor, and histone 2B. The roles these proteins play in HIV-1 infection are unknown at this time.

5.5 Conclusions

In the projects outlined in this paper, two-dimensional gel electrophoresis coupled with mass spectrometry was used to identify proteins that were sensitive to a treatment by comparison with controls. These studies compared the protein expressions of the proteome at the time of isolation. Despite the limitations confronted when using proteomics techniques, when combined with other complementary technologies, they have enormous potential to provide new insight

into biology. The ability to study complex biological systems in their entirety will ultimately provide answers that cannot be obtained from the study of individual proteins or groups of proteins.

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