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Genomic and Proteomic Techniques and their Application in Selenium Research

Huawei Zeng*

United States Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, North Dakota 58202-9034, USA

Abstract: Recent revolutionary changes in molecular biology have spawned the disciplines of genomics and proteomics that systematically generate and analyze the information about genomes, gene transcripts, proteins and their functions in a global, comprehensive manner. The applications of these approaches present tremendous opportunities in almost every aspect of bioscience research. One such opportunity concerns selenium, an essential trace element for humans and many other forms of life, which has been associated with reduced cancer risk. Interestingly, the biological activities of selenium as a nutrient, a cancer preventive agent, or even a toxicant, are dependent on the dose and the chemical form of the element. However, the molecular mechanisms by which selenium exerts these effects largely remain unknown. This article outline*s* the current status of genomic and proteomic techniques and their application in selenium research, particularly as it relates to the prevention of tumorigenesis.

INTRODUCTION

Selenium is an essential trace element for humans and many other forms of life (Stadtman,1996). In addition, selenium has been associated with reduced cancer risk. The anticancer effects of selenium in humans have been supported by a substantial body of persuasive evidence (Schrauzer, 1977; Ip, 1998) including animal studies (Finley *et al*. 2000), human epidemiological investigations (Combs *et al.*2001), and intervention trials (Clark *et al.*1996). The biological activities of selenium as a nutrient, a cancer preventive agent, or even a toxicant, are dependent on the dose and the chemical form of selenium (Ip *et al.* 1991). Most animal studies have used pharmacologic doses of selenium (> 2 mg/kg) in cancer chemoprevention (Ip, 1998) but low selenium intake has also been shown to increase cancer susceptibility when compared with physiologic doses in human and animal studies (Clark *et al.* 1996; Davis *et al.* 2002). The physiological role of selenium involves antioxidant protection because many selenocysteine-containing proteins are antioxidant enzymes such as the glutathione peroxidases and the thioredoxin reductases. However, much remains to be learned about the essential nutritional role of selenium and the mechanisms by which selenium prevents tumorigenesis. Studies have demonstrated that apoptosis is an important mechanism for the anticancer effects of selenium, particularly at pharmacologic levels (Ganther, 1999). A full understanding of that and other possible mechanisms will require a comprehensive and systematic analysis of gene transcripts and their protein products. Founded on automation, bioinformatics and other recent technologies, genomics and proteomics are the disciplines that systematically generate and analyze the information about genomes, gene transcripts, proteins and function in a global, comprehensive manner. The application of genomics and proteomics to selenium research holds a great potential in identifying seleno-proteins and selenium related pathways. A better understanding of selenium-related molecular targets and pathways is key to finding potential therapies or even to reducing cancer incidence using selenium compounds.

IDENTIFYING NEW SELENOPROTEINS AND THEIR FUNCTIONAL CHARACTERISTICS

There are three groups of known selenium-containing proteins. The first group consists of proteins in which dietary selenomethionine is deposited nonspecifically in place of methionine. The second group consists of proteins in which selenium is non-covalently attached with high affinity. The third group consists of selenoproteins that contain selenium in the form of genetically encoded selenocysteine in which a selenium atom is found in place of sulfur (Stadman, 1996). Evidence for the nutritional essentiality of selenium includes the fact that seleno-proteins have been identified and characterized in bacteria, archaea, plant and eukaryotes (Castellano *et al.* 2001; Fu *et al.* 2002), and selenoproteins appear to be particularly abundant in mammals: 22 have been found to date (Behne and Kyriakopoulos, 2001).

Current knowledge of selenoproteins was obtained largely from experiments in which animals or cultured

*Address correspondence to this author at the USDA, ARS, GFHNRC, P. O. Box 9034, Grand Forks, ND 58202-9034, USA; Tel: 701-795-8465, Fax: 701-795-8220; E-mail: hzeng@gfhnrc.ars.usda.gov

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cells were labeled with ^{75}Se, and the ^{75}Se-containing proteins were identified and purified by chromatographic or gel electrophoretic separation, and subsequently, digested and sequenced. Then, cDNA was cloned by the DNA polymerase chain reaction (PCR) method or identified through computer searches of the sequence in the expression sequence tag (EST) databases. More recently, *in silico* identification at the genome-scale has proven to be another powerful approach in identifying new selenoproteins, their expression and functions. This approach is based on the study of selenoprotein translation. Selenocysteine (Sec) is the 21st amino acid to be recognized as being encoded onto proteins, with UGA as its genetic codon (Stadman,1996). Sec is typically located in enzyme active centers where it is essential for catalytic activity of the selenoproteins. Although the characterized selenoproteins lack any common amino acid motifs or patterns, selenoprotein genes do have a common mRNA stem-loop structure, designated the Sec insertion sequence (SECIS) element. The SECIS is a hairpin residing in the 3'-untranslated region of selenoprotein mRNAs that is essential for the read- through of the UGA selenocysteine codon, otherwise, UGA functions as a stop codon (Stadman, 1996). A computer program for identification of SECIS elements in nucleic acid sequences has been developed (Kryukov et al ., 2002; Castellano *et al.* 2001). This involves four major steps: 1) SECIS element primary sequence search, 2) SECIS mRNA secondary structure search, 3) assessment of the thermodynamic stability of the predicted secondary structure, and 4) cDNA open reading frame (ORF) analysis.

To gain further insight into newly identified selenoproteins, several different experimental approaches are commonly used. First, for the detection of selenium in the expressed proteins, cultured cells are transfected with the plasmids encoding newly discovered cDNAs such as SelT and SelR. For this approach, transfected cells are grown in the presence of ^{75}Se, and ^{75}Se-labeled proteins are resolved by polyacrylamide gel electrophoresis and visualized with a PhosphorImager (Kryukov *et al.* 1999; Castellano *et al.* 2001). Second, to study the function of new selenoproteins, the gene knockout mouse is useful. For example, mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide (de Haan *et al.* 1998). Third, in addition to gene knockout, at the cellular level, small interfering RNA (siRNA) provides a new approach for elucidation of gene function in cultured mammalian cells (Harborth *et al.* 2001). RNA interference is a sequence-specific, post-transcriptional gene silencing mechanism. In combination with modern screening technology, siRNAs may become a widely useful tool for analysis of selenoprotein gene function with high throughput. Fourth, the identification of single nucleotide polymorphic sites (SNPs) that occur within selenoprotein genes can provide useful information, as such SNPs can have important functional consequences. For example, the 3'- untranslated region polymorphisms in human 15-kDa selenoprotein result in changes in selenocysteine incorporation into protein, respond differently to selenium supplementation, and may even influence the risk of cancer development (Kumaraswamy *et al.* 2000; Hu *et al.* 2001).

COMPREHENSIVE GENE TRANSCRIPT ANALYSIS

Identification of selenium-related molecular targets and delineation of their pathways may be keys to finding potential therapies or even to preventing cancer. Access to the full sequence of the human genome and expression sequence tag (EST) databases offers tremendous opportunities in this regard. The integration of robotic technology and molecular biology has resulted in the production of gene arrays. Methods for microarray fabrication include spotting of DNA onto nylon membrane or glass slides by robots with pins or ink jet printers (Relogio *et al.* 2002). There are two major gene array configurations; those consisting of chemically synthesized DNA chains (oligonucleotides) and those consisting of DNA fragments obtained from reverse-transcribing messenger RNAs (cDNA) (Chee *et al.* 1996; Schena *et al.* 1996). cDNA arrays are often used in RNA expression analysis, while oligonucleotide arrays are additionally used for sequence analyses. Gene arrays can be divided into three categories based on their gene density: 1) DNA macro-arrays contain a few dozen up to a hundred genes, 2) DNA semi micro-arrays contain a few hundred genes, 3) DNA micro-arrays contain thousands of genes. DNA macro-arrays which do not require special equipment are often used in gene expression analysis of a specific signal pathway, while DNA micro-arrays are used in global, comprehensive analyses of gene expression.

Although a species has only one genome, the gene expression profile or proteome is unique for every cell type and tissue. Furthermore, this unique gene expression profile or proteome may be affected by various environmental and developmental factors. Thus, the gene array is a powerful tool for screening gene expression in a comprehensive manner, and has proven valuable for discovering selenium-related molecular targets. A comparison of the expression pattern for different gene transcripts can detect the important changes at gene transcription, which can be followed up with more detailed biochemical analyses. It is essential that one should determine the chemical form and dose of selenium depending on the experimental goal before applying genomic and proteomic techniques to selenium-exposed cells. For example, in mammalian cultured cells, ~ 3 - 5 μmol/L selenite induces cell cycle arrest and/or apoptosis which may be due to the changes in cellular redox state. In contrast, selenite is an essential trace nutrient at ~ 50 - 250 nmol/L for optimal growth of most mammalian cell lines. At low concentrations of selenite, one may detect the increase of cell growth, promotion of cell cycle progression and up-regulation of selenocysteine-containing enzymes such as glutathione peroxidases. The changes of secondary effects such as cellular redox state are highly unlikely because the cultured medium has a buffering capability for nmol/L but not μmol/L selenite. Therefore, one can identify the biologically relevant changes that account for nutritional

role of selenite in cultured cells when selenite is at nmol/L level (Zeng, 2002).

Understanding the effect of chemical forms and doses of selenium on cell cycle arrest and apoptosis is a critical step in determining the cancer preventive efficacy of a given selenium compound. A comparison of the effect of different chemical forms and doses of selenium is required to distinguish mechanistic observations from those that would be anticipated for any cytostatic or pro-apoptotic compound. Many selenium compounds are used for investigating the cancer chemopreventive action of selenium *in vitro.* Apoptosis induction is a major mechanism mediating the anticancer activity (Ganther, 1999). For example, previous work indicated that distinct cell death pathways are likely involved in apoptosis induced by the CH_3SeH and the hydrogen selenide pools of selenium metabolites. Methylseleninic acid (MSeA) is a novel penultimate precursor of the putative critical anticancer metabolite CH_3SeH (Jiang *et al.* 2001). On the other hand, sodium selenite is a representative of the genotoxic selenium pool which would be anticipated for the cytostatic or pro-apoptotic effect. Both MSeA and sodium selenite start to induce apoptosis at ~ 3 - 5 μmol/L for cultured cells. Therefore, if one is interested in identifying the distinct mechanism of apoptosis induced by MSeA, sodium selenite can be used as a reference for comparison of genomic and proteomic data. Such information may provide the foundation for the selection of specific selenium compounds and doses in nutritional and pharmacological applications.

The utilization of gene arrays in selenium research is still in its early phase, but gene transcript profiling has already been showing great potential to deepen our understanding of selenium-related molecular pathways. DNA array analyses of selenium deficiency *verus* adequacy in cultured HL-60 cells, mouse intestine, and rat liver revealed that low selenium status results in activation of genes involved in DNA damage, oxidative stress , cell cycle control and detoxification (Zeng, 2002; Rao *et al.* 2001; Fischer *et al.* 2001). A side-by-side hybridization (Fig. **1**) shows a decrease in the c-Myc mRNA level in selenium-deficient, compared with selenium-treated cells. Previous studies indicated that methylselenic acid (MSA), se-methylselenocysteine (MSC), -glutamyl-Se-methyselenocysteine (GGMSC) are excellent tools for investigating the cancer chemopreventive action of selenium *in vivo* (Dong *et al.* 2001, 2002). With cDNA microarray analysis, Dong and co-workers have proposed a tentative signaling pathway mediating the outcome of selenium-induced cell cycle arrest and apoptosis (Dong *et al.* 2001, 2002).

BEYOND GENOMICS, TO A COMPREHENSIVE ANALYSIS OF GENE EXPRESSION: PROTEOMICS

Although gene transcript profiling offers us tremendous insights into the regulation of gene transcription, additional information is needed to understand biological function. At the cellular level, biological function is determined by proteins, not by DNA or RNA. Several studies have shown that mRNA levels do not necessarily correlate with the corresponding protein concentration or with cellular function (Gygi *et al.* 1999a). In addition, RNA splicing and post-translational modifications such as phosphory-lations greatly affect protein activity. Furthermore, protein conformation/tertiary structure, post-translational modifications, protein-protein interactions and the micro-environ-ment are

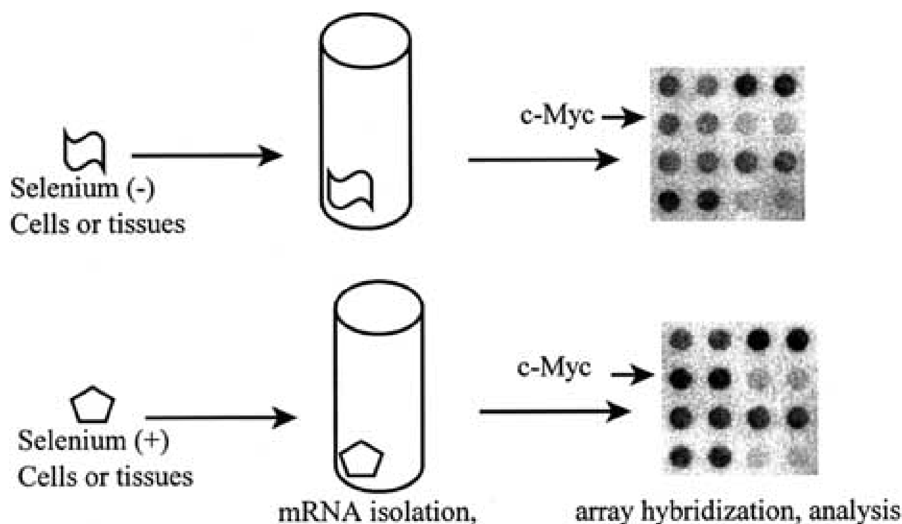

Fig. (1). The effects of selenium status on gene expression profiling

The cDNAs are synthesized from mRNA samples isolated from cells or tissues with different selenium treatments. These cDNAs are then labeled with isotope or fluorescence, and hybridized with gene arrays. A scanner would read the intensity of isotope/fluorescence at each spot, which would represent the level of gene transcripts.

determinant factors in cellular function. For these reasons, the comprehensive analysis of cellular protein profiling is a great challenge, and there is not at the moment an amplification system for proteins analogous to the DNA polymerase chain reaction. The significance of comprehensive analysis of cellular protein products, interaction, and function is apparent. Recently, several techniques have emerged and advanced to the point where comprehensive analysis of proteins is achievable. The following discussion will focus on important experimental approaches that emerged from recent proteomics advances, which should have great potential applications in selenium research.

(1). Direct protein-protein interaction

Many cellular functions rely on interactions between proteins. A prime example is the gene transcription process, requiring that transcription factor, RNA polymerase and ancillary proteins orderly assemble and form the protein machinery which initiates transcription (Sanders *et al.* 2002). A full understanding of selenium's cellular functions will require a complete picture that includes not only the protein players but also the network of protein interactions. A yeast two-hybrid system has been used to detect protein-protein interaction as well as to analyze the affinity of these interactions (Fields and Song, 1989). Most eukaryotic transcriptional activators consist of two discrete domains: the DNA-binding domain (DNA-BD) and an activation domain (AD) (Fig. **2**). When the DNA-BD binds to the specific promoter sequence, the AD contacts the RNA polymerase II complex to initiate the downstream gene transcription. The DNA-binding domain and the activation domain are two independent modules; each domain still functions even when fused to other protein. The two-hybrid system has been shown to be extremely useful for placing a protein of unknown function within a functional context, thereby providing information about a putative role of the uncharacterized protein. A uniquely expressed protein library can be made from cells or tissues of interest through a expression vector. In principle, a selenoprotein may be used as a "bait" protein to screen against this expressed protein library to identify proteins that directly interact with the selenoprotein *in vivo* (Fig. **2**).

(2). Differential analysis of " Proteograph"

In the past, most laboratories studied proteins or signaling pathways one at a time. Recent revolutions in molecular biology have sparked a surge in interest in the global analysis of gene expression at the protein level. Currently, reversed phase- high performance liquid chromatography (RP-HPLC), matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS), and two-dimensional gel electrophoresis (2DE) are the main approaches in identification, distribution, and characterization of selenoproteins (Kyriakopoulos *et al.* 2002; Gulesserian *et al.* 2001). With recent advances in electrophoresis, computerized imaging, protein digestion and mass spectrometry, protein molecules can be individually identified by comparing their "fingerprints" with large protein databases (Fig. **3**). For example, sequence analysis of the peptides produced by proteolytic digestion, performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS), confirmed the existence of a selenocysteine residue in *Chlamydomonas reinhardtii* glutathione peroxidase (Fu *et al.* 2002). However, the 2DE/MS/MS methodology is labor intensive, has a relatively low throughput, and is not well suited for the analysis of protein mixtures containing low-abundance proteins. More recently, the Isotope-Coded Affinity Tag (ICAT) reagent method has enabled the concurrent quantification and

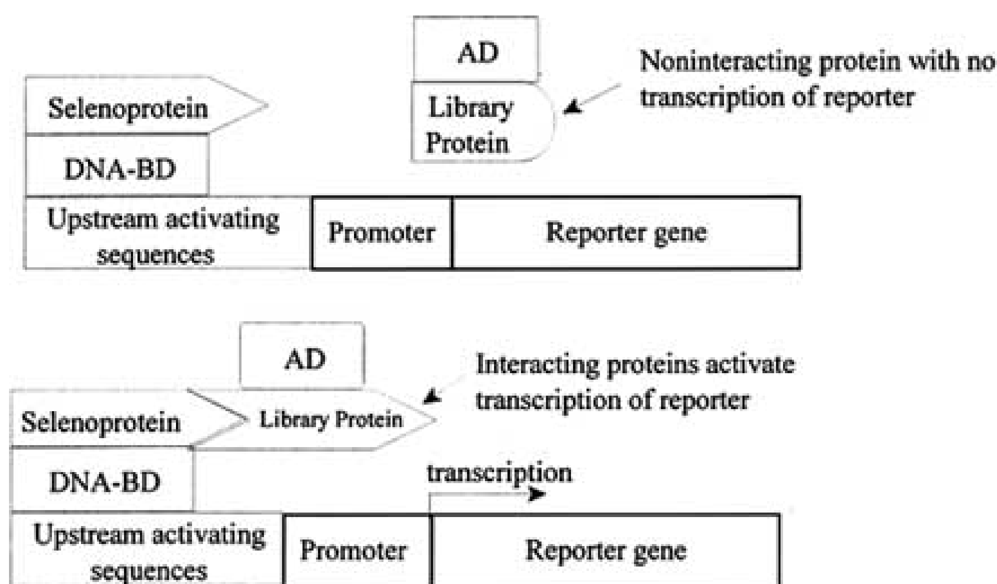

Fig. (2). General schema for detection of direct protein-protein interaction by the two-hybrid assay

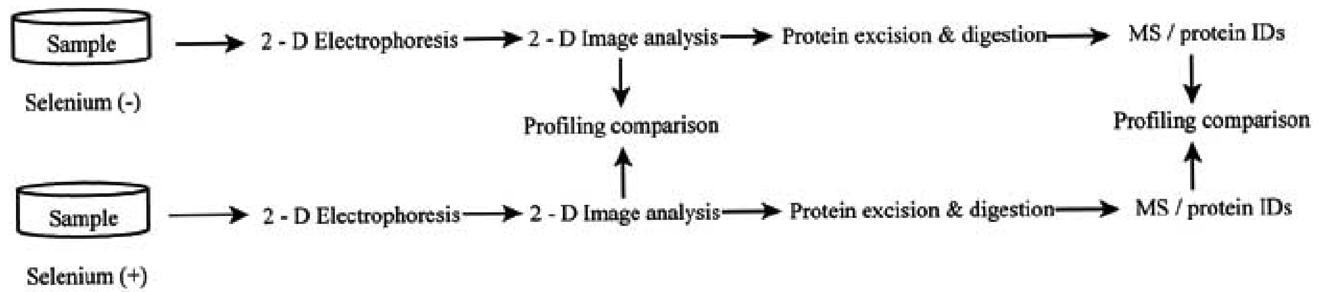

Fig. (3). The 2 D gel and MS based analysis strategy

The pre-fractionalized protein samples from different selenium statuses are subjected to isoelectric focusing/SDS-PAGE separation (2 D gel), and fluorescent/silver staining. An imaging device is used to detect protein spots on the stained gel, to compare many gels simultaneously, and to identify significant changes in protein expression. These protein spots are then excised and digested. Then, MALDI mass spectrometry is performed to determine protein identification based on the recorded sequence information in protein databases.

identification of expressed proteins in complex mixtures (Gygi *et al.* 1999b). The heavy or light ICAT reagents are coupled to the cysteine residues in the proteins of samples from two treatments, respectively, and the relative abundance of the proteins in the two samples is determined by comparing the intensity of the identical peptide peak pair as defined by the isotopic difference in the light and heavy reagents. This approach can be used to study the effect of selenium on differential "proteographs": two cell or tissue samples can be treated with different doses of selenium, and then be mixed, digested, and analyzed in mass spectrometers (Fig. **4**).

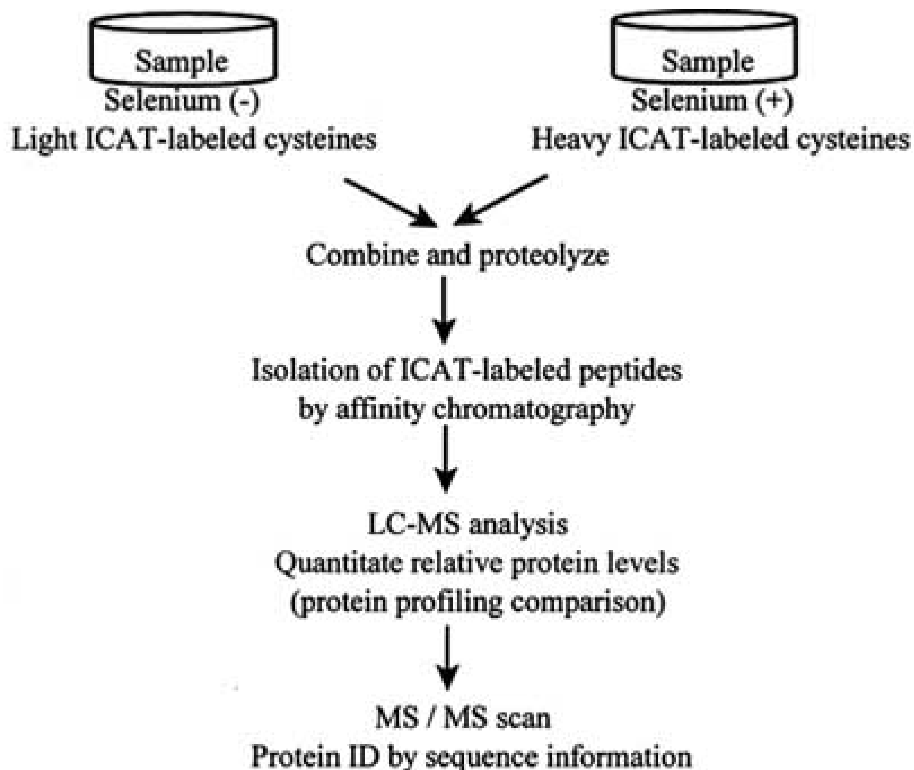

Fig. (4). The isotope-coded affinity tag (ICAT) - labeled MS based analysis strategy

Two protein mixtures treated with different doses of selenium are labeled with the isotopically light and heavy ICAT reagents, respectively. The ICAT reagent is covalently attached to each cysteinyl residue in every protein. The two protein mixtures are combined and proteolyzed to peptide. Subsequently, the ICAT-labeled peptides are isolated by affinity chromatography because the ICAT incorporates a biotin molecule. The ratio of the isotopically light and heavy peptide pair is determined by mass spectrometry, which gives the ratio between the original proteins from two selenium statuses. The protein is further identified by computer-searching of recorded sequence information against protein databases.

The protein chip technology is another novel and powerful tool for high throughput assays of protein expression profiling, enzyme activity and protein-protein interaction. The enormous interest in microarray-based assays comes from the work using DNA chips. In principle, binding assays such as nucleic acid-protein, protein-protein, ligand-receptor, enzyme-substrate, ion exchange and metal affinity can be carried out in a microarray format. This area is still in its infancy because of the inherent complexity of protein molecules. Recently, a ProteinChip(R) array technology, surface enhanced laser desorption ionization (SELDI) has been developed by Ciphergen Biosystems (Fremont, CA). This method involves a unique combination and miniaturization of both SELDI and ProteinChip technologies on a single, unified platform. Briefly, a complex mixture of proteins, as from cells or body fluids, can be reduced to sets of proteins with common properties by binding the sample to chips with differing binding affinity in parallel and in series. After the chips are washed to remove unbound proteins, the bound proteins are read in a time-of-flight mass spectrometer (TOFMS). The resulting spectra give a multi- dimensional binding picture on the basis of different types of interaction. The process, known as surface- enhanced laser desorption/ionization (SELDI), allows for the analysis of proteins directly from biological and clinical samples at femtomole levels (Fung *et al.* 2001; Wellmann A *et al.* 2002). Once a peak of interest has been detected, on-chip digestion with proteolytic enzymes followed by analysis of the peptide sequences can yield the information on protein identification. Further advances in ProteinChip arrays promise a vast potential of fast identification of selenium-related molecular targets both in nutritional and pharmacological investigations.

In summary, genomics and proteomics provide global views of gene expression and their cellular functions. The examples given above show that genomic and proteomic technique are already useful tools for selenium research , and further application of these techniques will certainly advance our understanding of the essentiality of selenium and basic mechanisms by which selenium prevents tumorigenesis.

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