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Journal of Chromatography B, 722 (1999) 203–223

JOURNAL OF CHROMATOGRAPHY B

Review

Proteome analysis
II. Protein subcellular redistribution: linking physiology to genomics via the proteome and separation technologies involved

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Abstract

While annotated two-dimensional (2D) gel electrophoresis databases contain thousands of proteins, they do not represent the entire genome. High-molecular-mass proteins in particular are conspicuously absent from such databases. Filamin is prototypical of this class of proteins since it is a dimer with relative molecular mass (M_r) of 520 000 containing at least 240 potential phosphorylation sites. Filamin is not readily separated by current 2D procedures, and is difficult to study with respect to cycles of phosphorylation–dephosphorylation. Novel technologies are needed to identify biochemical pathways impinging upon such targets. The success of immunofluorescence microscopy as a research tool can be attributed in part to the fact that proteins redistribute in response to a variety of physiological stimuli. Comparable quantitative methods are required in proteome analysis. Three components are necessary for development of an approach that is capable of screening for protein redistribution events: (1) subcellular fractionation, (2) protein labeling and (3) data acquisition. An integrated approach is presented that utilizes differential detergent fractionation combined with reversible, luminescent protein stains and analytical imaging for high-throughput analysis of signal transduction events leading to protein subcellular redistribution. The procedure has been successfully implemented to rapidly define key second messenger pathways leading to endothelial cell junctional permeability and to guide in the design of a new family of peptide-based anti-inflammatory drugs.

Keywords: Reviews; Proteins; Proteomes

Contents

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00309-0

1. Introduction

1.1. Overview of proteome research

Large-scale research efforts such as the Human Genome project and cancer research continue to rely upon quantitative analysis of both structure and function of genes and gene products (proteins), with the detection of changes between normal and diseased states being of paramount importance. The United States Congress launched the Human Genome Project in 1988 by appropriating funds to both the Department of Energy (DOE) and the National Institutes of Health (NIH) in support of research efforts to determine the structure of complex genomes. Gene sequence information has been rapidly accumulating in database repositories since. In 1990, the US National Cancer Institute (NCI) launched a separate program to screen more than 60 000 compounds against a panel of 60 human cancer cell lines for determination of potential target molecules and modulators of activity [1,2]. The 50% growth-inhibitory concentration of each drug in the cell lines provides useful clues about drug action and resistance when correlated with the expression levels of various proteins within the cells. Integration of molecular approaches like the Human Genome Project with functional approaches like the NCI drug screening program is key to the burgeoning field of functional genomics, of which proteomics is an important component.

Research at the protein level has lagged behind DNA- and mRNA-based approaches due to the complexity of the technology required to separate, analyze and identify the thousands of proteins encoded by a typical genome [3,4]. Since DNA and mRNA are chemically quite homogeneous compared with proteins, genomics techniques such as DNA sequencing, mRNA isolation, cloning and PCR amplification are relatively easy to automate and readily applied to any gene without much modification. Proteins are chemically heterogeneous due to their diverse functional roles in cells and consequently techniques that work for one protein are often unsuitable for others. It is expected that the entire complement of human genes will be sequenced within the next few years, allowing a series of all human transcripts to be attached in arrays on a hybridization detector for quantitative detection of all transcripts in a single screen. The analysis of biological processes at the DNA/mRNA level will essentially be complete upon development of such genomic capabilities and attention must then turn to protein function as it relates to physiology itself.

Proteome research endeavors to examine the total protein complement encoded by a particular genome and to address biological problems that can not be answered by examination of nucleic acid sequence alone [5–8]. With regard to a global understanding of phenotypic responses to phenomena such as disease, environmental stresses, embryogenesis and aging, the total amount of a particular gene-product (mRNA or protein) is often of secondary importance compared to such parameters as the gene-product's rate of synthesis, rate of degradation, functional competence, degree of post-translational modification, sub-

cellular distribution and physical interactions with other cell components. A single gene may generate a single protein, no protein (pseudo-gene) or a number of proteins (through post-translational modifications, proteolytic cleavage and differential exon splicing) [9]. Proteome analysis provides data on when or whether a predicted gene product is actually translated, its relative concentration compared with other gene-products, as well as the level and type of post-translational modification it may undergo [9]. Proteome technology is expected to complement molecular biology approaches such as DNA sequencing, chromosomal linkage analysis, direct or subtractive hybridization, expressed sequence tags, differential display, microarray assay for gene expression and serial analysis of gene expression (SAGE) in addressing complex biological problems [9–12].

1.2. Proteomics: a convergence of disciplines

The field of proteomics arose from a convergence of three primary disciplines; two-dimensional (2D) gel electrophoresis, protein microchemistry and bioinformatics. Important milestones that facilitated the genesis of present day proteome analysis include development of 2D gel electrophoresis in the mid 1970s, silver staining, immunoblotting and computerized image analysis in the late 1970s through early 1980s, immobilized pH gradients in the mid 1980s, improvement of Edman-based protein sequencing in the late 1980s and adaptation of mass spectrometry to protein analysis in the early 1990s [5,9,10,13,14]. Recognition that simple databases of gene sequence information do not provide insight into functional (phenotypic) aspects of diseases has spurred interest in integrating these technologies for the examination of complex disease processes.

Two-dimensional gel electrophoresis, the separation of proteins in the first dimension according to their charge by isoelectric focusing (IEF) and in the second dimension according to their relative mobility (R_F) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is capable of simultaneously resolving thousands of proteins as constellation patterns of spots [15]. While conventional carrier ampholyte IEF gels utilize thousands of low-molecular-mass polyamino–polycarboxylic acids of differing isoelectric points to generate a pH gradient upon application of a voltage, the newer immobilized pH gel (IPG) technology utilizes a physically cast gradient of acidic and basic acrylamido derivatives covalently affixed to the polyacrylamide matrix during gel polymerization in order to form the pH gradient [16]. For broad range pH 4–8 gradients, the two techniques are essentially equivalent with respect to reproducibility between laboratories and resolution of protein spots, particularly when a thread support is incorporated along the length of the carrier ampholyte gel prior to polymerization [17–19]. Currently, both carrier ampholyte and IPG IEF resolve high-molecular-mass proteins rather poorly, but the IPG technique is particularly unsatisfactory as large proteins tend to adsorb to charged binding sites in the IEF gels and never enter the second dimension gel [9].

Efforts to identify proteins in 2D gels have proceeded for decades with comprehensive databases of specific cell types being constructed utilizing batteries of antibodies in conjunction with Western blotting. Results of such gargantuan enterprises have been painstakingly tabulated in monolithic 60 to 90 page publications authored by 20 or more scientists. For example in December, 1990, five years before the purported ‘‘proteome revolution’’, the laboratory of Dr. Julio Celis published a comprehensive 2D gel protein database for transformed human amnion cells containing about 100 proteins identified by immunoblotting, 80 identified by co-migration with purified proteins and another 20 proteins identified by Edman-based sequencing [20]. The broad field of post-separation protein microchemistry is key to linking gel separated proteins to a genome, however, and has largely replaced immunoblotting and protein co-migration as definitive in establishment of protein identity [8,21–33]. The majority of protein identifications are currently achieved by automated Edman-based protein sequencing which determines short, contiguous stretches of amino acid sequence that are suitable for searching genome databases [34]. Amino acid compositional analysis and peptide mass fingerprinting using mass spectrometry allow higher throughput identification of proteins and are increasingly displacing the costly and slow Edman degradation procedure [35–41]. Mass spectrometry-based analysis of proteins from 2D gels is usually performed by electrospray ionization mass spectrometry

(ESI-MS) or matrix-assisted laser desorption mass spectrometry (MALDI-MS) [42,43]. Often combinations of amino acid composition and peptide mass analysis techniques are utilized along with the protein's apparent molecular mass and isoelectric point to increase confidence in the accuracy of protein identification [26,44,45].

Bioinformatics, literally the use of computer technology in the analysis of biological data, has played a critical role in two fundamental aspects of proteome research; the development of 2D gel and genomic databases. Computer-assisted image analysis combines machine vision, digital image processing and data processing for interpretation of the complex patterns of spots generated on multiple 2D gels [46,47]. Images of electrophoresis gels are commonly obtained using charge-coupled device (CCD)-camera systems or document scanners. After image acquisition, image noise is removed, spot boundaries are detected, the amount of protein in each spot is established, and the coordinates of each spot are determined. The images are then linked together into a gel database and relevant spots are located by user specified queries [46,47].

With the complete sequencing of more than a dozen prokaryotic and one eukaryotic genome, bioinformatics has become increasingly important in linking long lists of genes to biological responses [48–50]. The establishment of interrogable genomic database repositories allows protein sequence, amino acid composition or peptide mass data to be utilized in the identification of genes. Several laboratories are generating comprehensive, annotated 2D protein databases that are accessible on the World Wide Web and linked to genome databases (see for example: http://biobase.dk/cgi-bin/celis and http://www.expasy.ch) [4]. Efforts are also under way to curate and organize information about proteins from the scientific literature and link the formatted data with genomic databases, and literature repositories such as PubMed [50].

1.3. Deficiencies in the proteome approach

Proteome analysis, as currently implemented, falls short of examining the total complement of proteins expressed by a genome. This is due in part to the fact that all proteins are not simultaneously expressed at any given point in time. For example, certain proteins are only expressed at specific stages of the cell cycle. Technological issues surrounding the principal methodology for resolving proteins, 2D gel electrophoresis, also prevent examination of the entire repertoire of expressed proteins. These issues include failure to detect proteins present in low copy number, co-migration of proteins, poor resolution of hydrophobic proteins and compromised ability to resolve very basic and high-molecular-mass proteins [13,51].

In the pre-genome era of the late 1970s and early 1980s, the total number of proteins encoded by a typical cell was estimated to be 5000 to 20 000 based upon the number of mRNA transcripts that could theoretically be produced due to the informational RNA complexity of polysomes [52]. HeLa cells were thought to contain about 23 500 poly (A)-containing mRNA while mammalian brain was estimated to contain about 150 000 poly(A)-containing mRNA [53,54]. The yeast genome is now known to contain 6340 open reading frames and though the precise number is still a few years away, the human genome is currently estimated to contain approximately 50 000 open reading frames [10,55].

Though high-resolution 2D gel electrophoresis is currently better able to resolve thousands of proteins from complex mixtures than other analytical techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), it can not detect a complete proteome. Every spot on a 2D gel does not represent a different open reading frame. Post-translational modifications such as phosphorylation, glycosylation, acetylation, ADP-ribosylation and proteolytic cleavage could easily increase the total number of different protein species present in complex eukaryotes to 200 000. In addition, some protein modifications, such as oxidized methionine and acrylamidated cysteine residues, occur during sample preparation, electrophoretic separation and staining. Perusal of annotated 2D databases on the World Wide Web emphasizes that every spot on a 2D gel does not correspond to a unique open reading frame. Albumin appears as six spots, fibrinogen gamma-A chain as five spots, apolipoprotein A-1 as five spots, ceruloplasmin as nine spots, serotransferrin as 22 spots, heat shock protein-60 as five spots, protein disulfide isomerase

as four spots, and catalase as four spots (http://www.expasy.ch). Sadly, a high-resolution 2D gel containing 3000 spots may only display a small percentage of the total number of different protein species present in a typical eukaryotic organism.

This review focuses upon strategies to evaluate the high-molecular-mass proteins that are normally disregarded by proteome researchers. Even the supposedly ''new and improved'' 2D gel chemistries, employing immobilized pH gradients, are deficient in a long list of abundant proteins, examples of which are listed in Table 1. The majority of these proteins are lost due to failure to enter the IEF gel or failure to subsequently be released by the IEF gel matrix for entry into the second dimension SDS–polyacrylamide gel. Proteins with relative molecular masses of greater than 180 000 are very poorly resolved by current technologies. Though macroporous polyacrylamide gels or agarose/polyacrylamide composite gels could possibly alleviate

Table 1
Examples of proteins not present on annotated 2D gel databases and hence not included in global proteome studies

Protein	Subunit molecular mass (M_r)
Ankyrins	220 000–440 000
APC tumor suppressor protein	300 000
ATM protein kinase	350 000
Bullous pemphigoid antigen	240 000
CD45	210 000
Clathrin	180 000
Collagens	180 000–240 000
Desmoplakins	210 000–240 000
Dynein	530 000
Dystrophin	430 000
EGF receptor	180 000
Fibronectin	260 000
Filamins	280 000
Fodrin	230 000–240 000
Insulin receptor	165 000
$1P_3$ receptor	300 000
MAP-2	280 000
Myosins	210 000
Nebulin	800 000
Neurofilament-H	200 000
Neuronal L1 receptor	200 000
N-methyl D-aspartate receptor	180 000
Nuclear factor Brm	180 000
Nuclear mitotic apparatus protein (NUMA)	240 000
p300 transcriptional coactivator protein	300 000
PDGF receptor	180 000
Plectin	450 000
Receptor-type protein tyrosine phosphatase β (RPTPβ)	250 000
Ras-GAP associated protein	190 000
Ryanodine receptor	560 000
Spectrins	220 000–240 000
Talin	230 000
Tensin	210 000
Thyroglobulin	350 000
Titin	3 000 000
von Willebrand factor	270 000

Two-dimensional gel electrophoresis fails to adequately resolve high-molecular-mass proteins. Cited molecular masses are estimates based upon migration in one-dimensional SDS–polyacrylamide gels.

some of the difficulties associated with separation of large molecular mass proteins, a fully functional 2D fractionation procedure incorporating these gels has not been adopted by the research community [56]. Instead, most 2D gel electrophoresis is performed utilizing a 12 or 12.5% SDS–polyacrylamide second-dimension matrix to minimize the high-molecular-mass region so that only the well resolved, low-molecular-mass proteins are displayed for consideration. This review highlights the need for future innovative technological developments in high-throughput protein separation methodologies, particularly for high-molecular-mass proteins.

2. From genome to complex function, shortcomings of a purely molecular genetic approach to disease processes

The assumption that the abundance of specific mRNAs dictates the abundance of the corresponding proteins is fundamentally incorrect. As such mRNA does not represent a reliable indicator of a cell's phenotype with respect to its current state of functional activity. This was demonstrated conclusively in a collaborative publication between a proteome company, Large Scale Biology and a genome company, Incyte Pharmaceutical [57]. Utilizing Transcript Image methodology and quantitative 2D electrophoresis, the correlation coefficient between mRNA and protein abundance in human liver was determined to be only 0.48, demonstrating that post-transcriptional regulation of gene expression is quite common in higher eukaryotes. Thus, while nucleic acid sequence-based methods are well suited for the discovery of genes as well as characterization of sequence and splicing variants, functional changes in cells are best ascertained utilizing the proteins that carry out these activities themselves. There are many documented instances of protein levels and activities changing without corresponding changes in the levels of their associated mRNA. Generally, these phenomena fall into two broad biological categories; rapid and slow biological events. Examples of each are discussed, drawing upon proteome projects the author has personally been involved with.

2.1. Slow biological events

Many biological responses occur on a time scale of months to years, too long to precisely monitor transcription and translation. Both mRNA and protein are continually being synthesized and degraded, thus final levels of protein are not easily obtainable by measuring mRNA levels. An illustrative example of this type of biological response is the slow, pathological growth of vascular smooth muscle cells (VSMCs) in atherosclerosis and hypertension [58–60]. Atherosclerosis is characterized by intimal migration and proliferation of VSMCs (hyperplasia) while hypertension is manifested by enlargement of preexisting VSMC (hypertrophy) within the media of the blood vessel. Though early induction of proto-oncogenes are readily determined by standard molecular biology techniques, it is more difficult to demonstrate a direct relationship between these early changes and the relatively long-term changes in protein metabolism associated with the diseases.

We undertook to examine phenotypic changes in hyperplastic and hypertrophic VSMCs through monitoring protein abundance by computer-assisted image analysis of silver stained 2D gels [47]. Platelet-derived growth factor (PDGF) causes hyperplastic growth while angiotensin II (Ang II) causes hypertrophic growth in VSMCs. Comparisons of cultured, quiescent VSMCs, with cells exposed to PDGF, or Ang II revealed four up-regulated proteins belonging to two families of molecular chaperones, heat shock proteins and protein disulfide isomerases. Additional proteins identified included elongation factor EF-1β, a component of the protein synthesis apparatus, and calreticulin, another molecular chaperone. The changes in protein expression are consistent with the transition from a quiescent to a proliferative phenotype being accompanied by a loss of differentiated properties (contractility) and the acquisition of proliferative properties (protein biosynthesis/folding). Significantly, most of the proteins identified by the proteome approach had never before been implicated in hyperplasia or hypertrophy, even when differential cDNA screening or subtractive libraries were used to examine a large number of transcripts.

Probably the single biggest disappointment to the

molecular biology collaborators associated with this three year project was that the proteins identified were all rather abundant, housekeeping proteins. No proto-oncogenes, transcription factors or stress activated kinases were uncovered in the screen of 1000 proteins. This tends to be the rule rather than the exception in 2D studies and undoubtedly is due to the use of total cell extracts in the analysis. Two-dimensional gel electrophoresis is most likely to provide data on the high copy number components of a sample. This has been elegantly demonstrated in studies of the yeast proteome [24]. Codon bias index (CBI) values discriminate between frequently and occasionally expressed genes. Nearly all of the 149 different proteins identified from 2D gels of yeast extracts have CBI values of 0.2 or greater. These proteins include glycolytic enzymes, heat shock proteins and protein synthesis factors. Few proteins with low CBI values (<0.2), such as protein kinases and transcription factors have been identified on the 2D gels.

2.2. Rapid biological events

While hyperplasia and hypertrophy occur over the course of years, other biological responses occur on a time scale of seconds to minutes without transcription or translation changing the abundance of protein products. A case in point is the increase in endothelial cell junctional permeability accompanying blood vessel inflammation [61]. Proteins at the membrane–cytoskeleton interface, such as non-muscle filamin (ABP-280), coordinate interactions critical to the maintenance of endothelial cell barrier function [62,63]. Bradykinin is a prototypical regulatory metabolite that modulates vasodilation, increases vasopermeability and produces pain. Filamin is concentrated at lateral margins of endothelial cells but redistributes from the membrane to the cytosol after exposure to bradykinin [64–67]. Bradykinin-induced filamin translocation requires activation of calcium/calmodulin dependent protein kinase II (CaM-PK II) [64] (Fig. 1). Filamin translocation does not always occur via the CaM-PK II second messenger pathway, however. In a model of reoxygenation injury, micromolar concentrations of H_2O_2 cause filamin redistribution in a calcium-independent manner [68–70]. Decreased cAMP-dependent protein kinase (cAMP-PK) activity and increased phospholipase D activity lead to H_2O_2-induced filamin translocation and permeability changes [69,70].

Filamin redistribution events are rapid, reversible and independent of abundance changes in mRNA or protein levels. Filamin subcellular redistribution is utilized as a model system to illustrate a proteomic approach to analysis of large proteins in the remainder of this review article. The dynamic nature of this high-molecular-mass protein has largely escaped detection by conventional proteome analysis methods despite the fact that filamin was first isolated about 25 years ago and is nearly as abundant as actin and myosin in most eukaryotic cells [71].

3. Subcellular fractionation schemes to monitor protein subcellular redistribution

3.1. Why monitor subcellular redistribution?

The success of immunofluorescence microscopy as a research tool can be attributed in part to the fact that proteins redistribute in response to a variety of physiological stimuli. Activation of numerous cellular regulatory pathways is accompanied by the translocation of key proteins from one region of the cell to another. Examples of proteins that translocate in response to cell activation include, calcium–calmodulin dependent protein kinase II (CaM-PK II), protein kinase C, protein kinase A, protein kinase N, annexin IV, MARCKS, phospholipase A2, protein phosphatase 1, protein phosphatase 2A, Src kinase, Rho, dynein, nitric oxide synthase, cortactin, paxillin, filamin, spectrin, calreticlin, heat shock proteins and myosin.

The protein kinase C family well illustrates the essential role subcellular redistribution plays in the control of signal transduction pathways (Fig. 2). Eleven different protein kinase C isozymes belonging to three subfamilies have been identified, yet only limited differences between isozymes with respect to substrate specificity as well as sensitivity to activators or inhibitors are apparent [72,73]. Isozyme-specific functions appear to arise from differential subcellular distribution of the enzymes.

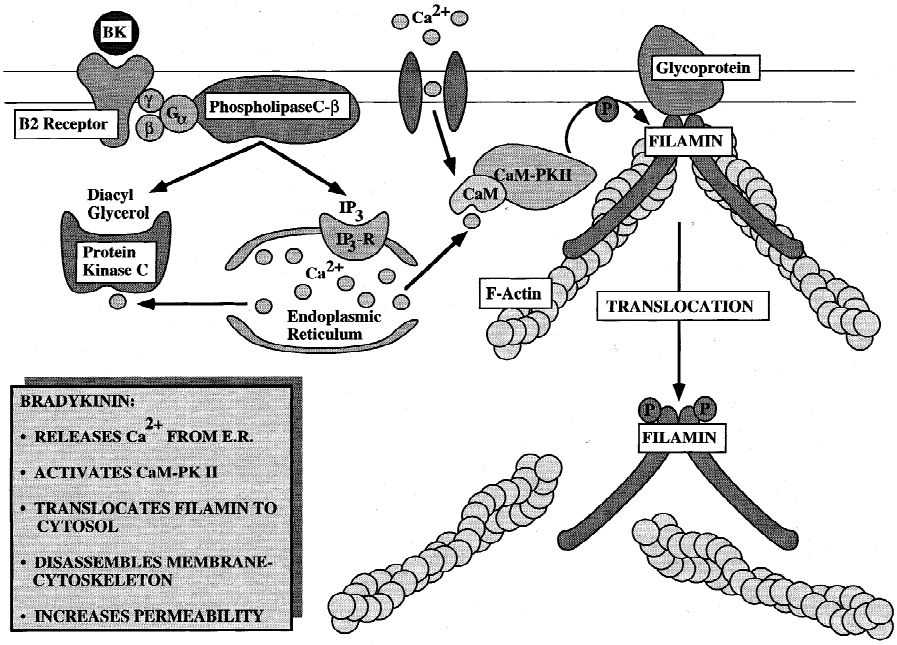

Fig. 1. Schematic diagram illustrating bradykinin-induced subcellular redistribution of filamin in endothelial cells. Bradykinin binds to its G-protein-coupled receptor (B_2) on the plasma membrane. This activates phospholipase C which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol. IP_3 subsequently binds to the IP_3 receptor (IP_3-R) on the endoplasmic reticulum which releases calcium. B_2 receptor activation also leads to calcium influx into the cell. Increases in intracellular calcium activate CaM-PK II which causes filamin translocation from the plasma membrane to the cytosol. This leads to the disruption of the dense peripheral band of F-actin that controls cell–cell apposition, endothelial cell retraction and increased intercellular permeability of the blood vessel. Filamin re-association with the plasma membrane leads to reorganization of the F-actin dense peripheral band and restoration of barrier function [64–67].

Inactive protein kinase C isozymes are thought to be anchored to various subcellular structures such as focal contacts, microfilaments, the golgi apparatus or perinucleus via receptors for inactive C kinase isozymes or RICKS. Lipid-derived second messengers, such as diacylglycerol cause translocation of isozymes from one compartment to another with targeting to the subcellular compartment controlled by another set of specific anchoring proteins, referred to as receptors for activated C kinase isozymes or RACKS. The redistribution of protein kinase C isozymes from RICKS to RACKS is thought to bring the enzymes in close proximity to specific substrates such as the myristoylated alanine-rich C kinase substrate, MARCKS. It is becoming increasingly evident that the intracellular localization of proteins involved in signal transduction cascades through regulated anchoring to intracellular structures is fundamental to a broad range of biological processes [74].

3.2. Advantages of monitoring subcellular redistribution compared to changes in phosphorylation status

Phosphorylation of proteins, the most frequent post-translational modification found in eukaryotic cells, is fundamental to the control of a multitude of cellular functions including transcription, proliferation, differentiation, and cell motility. Based upon 2D gel electrophoresis, approximately 30% of the proteins in a typical eukaryotic cell are phosphorylated [75,76]. Many subcellular redistribution events are triggered by changes in protein phosphorylation. For example, MARCKS (M_r of 80 000) contains two key domains that regulate the protein's

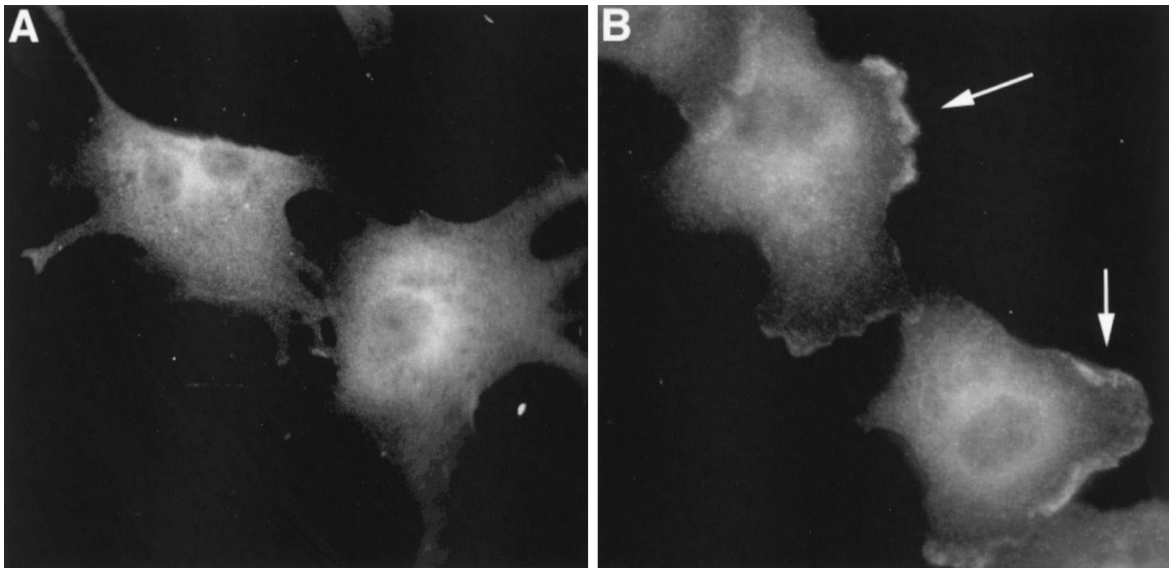

Fig. 2. Phorbol ester-induced redistribution of protein kinase Cα in bovine pulmonary artery endothelial cells. Protein kinase C is visualized utilizing a monoclonal antibody specific to the α isoform of the protein (Transduction Labs., Lexington, KY, USA) and Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Alexa 488-conjugated second antibody is employed because it is brighter and more photostable, yet utilizes the same excitation/emission wavelengths as fluorescein. (A) Untreated cells. (B) Cells exposed to 200 n*M* phorbol ester for 60 min. Arrows indicate redistribution of protein kinase Cα to the plasma membrane. A challenge to the separations sciences is to develop quantitative methods with spatial and temporal resolution comparable to immunofluorescence microscopy. Photographs courtesy of Dr. Chii-Shiarng Chen, Molecular Probes, Eugene, Oregon.

subcellular location [77]. The N-terminal myristoylated domain is responsible for protein association with the cytoplasmic face of the plasma membrane. A basic effector domain containing three serine phosphorylation sites for protein kinase C also contributes to the association of MARCKS with the negatively charged plasma membrane. Phosphorylation of MARCKS by protein kinase C leads to incorporation of nine negative charges in the effector domain. The ensuing electrostatic repulsion between the effector domain and the plasma membrane leads to the translocation of MARCKS to the cytosol. Dephosphorylation of MARCKS by protein phosphatases allows the protein to once again associate with the plasma membrane [78].

Though it is currently in vogue to study how protein kinases phosphorylate nuclear transcription factors and other protein kinases, it should be kept in mind that the cytoskeletal proteins are primary targets of phosphorylation cascades. Filamin is a dimeric protein consisting of two subunits (M_r 280 000) that contains numerous regulatory sites relevant to signal transduction [79–81]. Filamin contains 2647 amino acids, 360 of which are serine or threonine residues while the cytoskeletal protein ankyrin$_\beta$ contains 3924 amino acids of which 724 are serine or threonine residues. Ankyrin$_\beta$ (subunit M_r of 440 000) has about 220 while filamin has about 120 predicted sites for phosphorylation by various protein kinases per monomer [65,79,82]. Filamin isoforms isolated from unstimulated human platelets contain 18 to 40 phosphate groups per monomer, demonstrating that a significant percentage of the putative phosphorylation sites determined by examining consensus sequence motifs are physiologically relevant [83]. Direct evaluation of phosphorylation changes in relatively small proteins with few target sites is often fruitful [76]. Determining phosphorylation changes in high-molecular-mass proteins like filamin and ankyrin by conventional procedures such as peptide mapping, amino acid sequencing and in vitro mutagenesis of potential

phosphorylation sites is not currently feasible, particularly in large scale proteome projects that are designed to evaluate vast numbers of proteins simultaneously. Monitoring subcellular redistribution changes provides a rapid, high-throughput alternative to phosphorylation analysis for the examination of signal transduction events.

3.3. Methods for studying subcellular redistribution

An important challenge in separation sciences is to be able to measure protein movements occurring during cell activation. Two dimensional gel electrophoresis has already been extensively exploited to produce detailed maps of the static subcellular distribution of organellar proteins [23]. Despite their obvious regulatory significance, dynamic distributional changes in protein compartmentalization have not routinely been monitored quantitatively by combining subcellular fractionation with 1D or 2D gel electrophoresis. Though elaborate protocols for isolation of specific subcellular fractions such as plasma membranes, nuclei and golgi bodies are commonly utilized in biological research, surprisingly few procedures for the simultaneous fractionation of cells into several distinct compartments are available [84–86].

Conventional methods of subcellular fractionation such as differential centrifugation are compatible with 2D electrophoresis, providing that care is taken to remove salts from the sample prior to analysis. A number of subcellular fractionation schemes have been developed over the years for monitoring protein subcellular localization based upon this technique but many are unsuitable for high-throughput evaluation of redistribution events. Differential centrifugation combined with purification in sucrose, Percoll, metrizamide or Nycodenz gradients (rate zonal and isopycnic) is routinely used to isolate specific subcellular organelles [87]. Relatively large amounts of cells are needed for these procedures and separation of the organelle of interest typically requires 30 min to 2 h. This separation time is generally too slow for monitoring dynamic signal transduction events in cells. The cytoskeletal compartment is lost using these procedures as microfilament, microtubule and intermediate filament networks are disassembled during the initial cell lysis prior to centrifugation. Centrifugation procedures are also notoriously difficult to automate, an important consideration in proteome programs.

Fractionation by sequential extraction of cells or tissues with detergent-containing buffers allows partitioning of proteins into functionally distinct compartments that can readily be evaluated by 1D or 2D gel electrophoresis [85,86]. Unlike most other subcellular fractionation methods, detergent fractionation preserves the integrity of the cytoskeletal compartment. The regulatory significance of the cytoskeletal compartment is increasingly being recognized in processes such as endocytosis, exocytosis, mitosis, cytokinesis, chemotaxis, signal transduction and protein biosynthesis.

Countless laboratories have successfully utilized Triton X-100 detergent extraction techniques to fractionate cells into cytoskeletal (Triton-insoluble) and non-cytoskeletal (Triton-soluble) compartments [88–91]. Using a related technique, three subcellular fractions (cytoskeletal, membrane and cytosol) are obtained with Triton X-114 temperature-dependent phase separation [84,92]. Non-ionic detergents of the octylphenolpolyoxyethylene type such as Triton and Nonidet, separate into a detergent-rich and detergent-poor phase at their cloud point. Triton X-114 undergoes this transition at about 20°C while Triton X-100 undergoes a similar phase separation at 60°C. Integral membrane proteins partition with the detergent phase, cytosolic proteins with the aqueous phase and cytoskeletal proteins remain insoluble and hence are excluded from both phases. While the Triton X-100 procedure is rapid, only two fractions are obtained and subtle protein distributional changes are thus often missed. The Triton X-114 procedure provides more subcellular compartments for analysis but is arduous. Typically, 6–8 h are required for a total cell fractionation experiment.

Seven subcellular fractions corresponding to water-soluble, membrane, microfilament, intermediate filament, microtubule, polysome and nuclear proteins have been obtained utilizing a range of detergent and salt extraction procedures combined with centrifugation [85,93]. The later two fractions were further subfractionated into ribosomal proteins, elongation/initiation factors, chromatin proteins and non-chromatin proteins. The procedure is a set of discrete protocols that each start with intact cells,

rather than an integrated procedure for comprehensive subcellular fractionation of a single population of cells. Aspects of this extraction-based fractionation procedure laid the groundwork for a more recently developed differential detergent fractionation procedure that reproducibly partitions proteins into four compartments; cytosol, membrane/organelle, nuclear and cytoskeletal [86]. The method involves sequential extraction of cells with digitonin/EDTA, Triton X-100/EDTA and Tween-40/deoxycholate containing buffers (Fig. 3). EDTA enhances the rate and selectivity of extraction by detergents and prevents protein degradation by calcium-activated proteases. The steroidal compound digitonin complexes with cholesterol in the plasma membrane causing permeabilization and release of the cytosolic constituents of the cell. Digitonin concentration must be low (<0.1%) to prevent extraction of phospholipids leading to damage of mitochondria and endoplasmic reticuli [86]. Low concentrations of the non-ionic detergent Triton X-100 solubilize membrane and organellar proteins without compromising nuclear integrity. Substitution of Triton X-100 with Triton X-114 allows the membrane/organelle fraction to be further subdivided into integral membrane protein versus peripheral membrane/organellar protein fractions by temperature-induced phase partitioning [84]. The Triton X-100-insoluble pellet is further extracted with a combination of the non-ionic detergent Tween-40 and the weakly ionic detergent deoxycholate. This solubilizes nuclear proteins and proteins weakly associated with the cytoskeleton. The insoluble cell residue resulting from this final detergent extraction is enriched in a variety of cytoskeletal proteins such as intermediate filaments, nuclear lamins, fodrin and desmoplakins. The insoluble residue is conveniently solubilized in SDS/dithiothreitol. Many proteins are differentially distributed between more than one subcellular fraction utilizing the differential detergent fractionation procedure and we have determined that their subcellular distribution is altered upon cell activation [64–70]. One appealing aspect of this detergent extraction protocol is that the detergent buffers are all fully compatible with equilibrium and non-equilibrium 2D gel electrophoretic techniques.

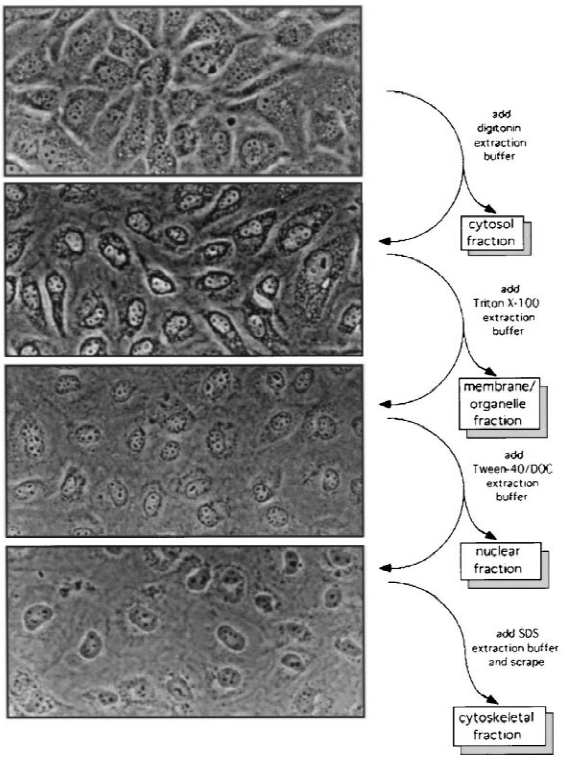

Fig. 3. Schematic diagram of differential detergent fractionation [86]. Phase contrast micrographs of bovine pulmonary artery endothelial cells are utilized to illustrate the procedure. Adherent cells are serially extracted in different detergent solutions to yield four subcellular fractions. Cytosol, membrane/organelle, nuclear and cytoskeletal fractions are obtained. Diagram courtesy of Dr. Eddie Chiang, Boston University.

Differential detergent fractionation has previously been validated in hepatocytes using seven marker enzymes, 11 cytoskeletal and 21 non-cytoskeletal protein markers [86]. Similarly, we have demonstrated the suitability of differential detergent fractionation in studies of endothelial cells and pericytes utilizing common marker enzymes and cytoskeletal proteins [64–70,94]. Detergent fractions may be resolved by 1D or 2D gel electrophoresis as well as after electroblotting to a membrane support (nitrocellulose or polyvinyl difluoride). Alternatively, high-throughput analysis of a protein of interest can be performed by vacuum slot-blotting detergent fractions without electrophoresis to nitrocellulose as arrays [95].

Differential detergent fractionation allows evaluation of complicated regulatory changes in protein dynamics due to the four compartments obtained and

fractionation can be achieved relatively rapidly. We have monitored changes in protein redistribution between the membrane and cytosol compartment that occur within 20 s of cell activation [64,68]. Differential detergent fractionation is simple, independent of time-consuming ultracentrifugation or cumbersome washing steps, amenable to low quantities of cells and fully compatible with electrophoretic procedures. The distributional changes in filamin, nitric oxide synthase, protein kinase C, NF-kappa-B and myosin we observe using differential detergent fractionation closely parallel changes observed by immunofluorescence microscopy ([64–70,94] and unpublished observations).

3.4. Potential pitfalls in studying subcellular redistribution

Certainly there is potential for experimental artifacts when evaluating translocation in subcellular fractions due to cell homogenization, extraction or centrifugation [96]. Some proteins may be inactivated or their subcellular distribution may change during the process of obtaining the different fractions. Additionally, recovery of a protein in a different compartment after cell activation does not always mean that the protein has literally translocated to that new compartment. Clustering of membrane receptors by an extracellular ligand, for instance, will lead to the association of specific integral transmembrane proteins with the underlying cytoskeleton [88–90]. While the membrane protein has not ''moved'' to the cytoskeleton, it has formed an association with that component of the cell and will likely partition with the cytoskeletal fraction during extraction of cells in a non-ionic detergent such as Triton X-100. It is probably best for the researcher not to get caught up in the semantics of the protein change and instead appreciate that the fractionation procedure has allowed recognition of the biological occurrence. The selective solubilization of different classes of lipids by detergents like Triton X-100 may also be problematic when assigning certain classes of protein to a subcellular fraction. The Triton-insoluble fraction is enriched in glycolipids, sphingolipids, cholesterol and some glycerophospholipids [97]. Proteins incorporating a glycosylphosphoinositol-lipid modification at their carboxyl-terminal end to anchor them into the plasma membrane are poorly solubilized by Triton X-100 and thus almost invariably partition to the cytoskeletal fraction [98].

Another note of caution is warranted regarding the post-fractionation method for analysis of proteins, 2D gel electrophoresis. Recent proteome scale evaluation of yeast proteins indicates that 2D gel electrophoresis portrays a biased picture of subcellular components [24]. While cytoplasmic, mitochondrial and endoplasmic reticulum proteins are well represented in 2D gels, nuclear, integral plasma membrane and golgi proteins are under-represented. Glycosylated proteins are also severely under-represented using current 2D procedures.

As stated in a recent publication: ''*Recognition that certain classes of proteins may not be detected under given conditions of sample preparation and electrophoresis is an important consideration in the design of proteome projects, and further research can be directed to finding conditions that lead to detection of these protein classes*'' [24].

Despite the potential for experimental artifacts, monitoring protein redistribution events in subcellular fractions is relatively simple, rapid and suitable for evaluating large numbers of proteins simultaneously. Protein redistribution events are usually reversible, allowing kinetic analysis of signal transduction events. Global patterns of protein redistribution produced by any ligand should serve as a unique profile of cell activation, just as phosphorylation changes can provide an identifying ''fingerprint'' of cell response [75].

4. Strategy for determining protein targets that undergo subcellular redistribution

4.1. Defining protein candidates by 1D or 2D electrophoresis

Two-dimensional gel electrophoresis is not ideal for integration into a high-volume protein translocation monitoring project since it is a low-throughput and labor-intensive method. For example, if the 60 cell lines specified in the NCI drug screening program are evaluated in duplicate with a single test agent and resolved into four subcellular fractions, a minimum of 480 2D gels are required [1]. Duplicate

sets of untreated, control cell lines bring the total number of gels to 960. Instead, 2D gel electrophoresis is better suited as a tool for exploratory analysis to identify key regulatory proteins used in subsequent screening programs. Additionally, in the absence of a satisfactory higher resolution method, high-molecular-mass proteins, like filamin and myosin, are best analyzed using differential detergent fractionation combined with 1D SDS–polyacrylamide gel electrophoresis. Typically, 4–15% gradient gels are well suited for this application. Once potential biomarkers for a particular signaling cascade are recognized by computerized image analysis, proteins can be identified by microchemical analysis and antibodies can be generated to the relevant polypeptides for use in the higher-throughput screening of large sample sets. Thus, proteome screening based upon protein translocation consists of two phases; target protein identification and implementation of the target in screening assays.

4.2. Colorimetric staining methods for detecting proteins

Target identification after subcellular fractionation requires a sensitive, total protein stain. There is no real consensus concerning whether chemical and proteolytic fragmentation procedures performed on membrane supports versus in polyacrylamide gels yield higher peptide recoveries for Edman-based sequencing and mass spectrometry [99]. Thus, rapid and sensitive protein detection procedures are required for both gels and blots. Coomassie Brilliant Blue R-250 (CBB-R) and colloidal Coomassie Blue G-250 (CBB-G) are widely utilized for the detection of proteins after PAGE [100,101]. CBB-R is capable of detecting as little as 30–100 ng of protein, while colloidal CBB-G is slightly more sensitive, detecting in the vicinity of 10 ng of protein [102]. Amido black is utilized extensively in the staining of electroblotted proteins on membrane supports, with a detection limit of 30 to 50 ng [99,103]. The sensitivity of post-separation protein microchemistry has increased to such an extent in recent years that common gel and membrane stains such as CBB and amido black are no longer capable of detecting all the minor proteins that could potentially be evaluated by proteome technology. In a recent study concerning the identification of proteins by solid-phase microextraction–capillary zone electrophoresis–microelectrospray-tandem mass spectrometry, duplicate protein samples were first separated by high-resolution 2D gel electrophoresis [103]. One gel was silver stained and the other electroblotted to nitrocellulose membrane and subsequently stained with amido black. The silver stained gel was used as a template to locate regions of the nitrocellulose membrane containing the low abundance proteins to be analyzed, as these proteins fell below the detection limit of the amido black stain.

Conventional silver staining is not compatible with Edman-based protein sequencing of proteins from gels and membranes, though mass spectroscopic analysis has successfully been applied utilizing modified approaches [99,104–107]. Silver staining of polyacrylamide gels allows detection of low nanogram amounts of protein but special care must be taken to avoid metal-catalyzed protein oxidation by chilling silver solutions to 4°C [105]. Fixation steps with glutaraldehyde should be avoided and only low concentrations of formaldehyde should be employed during development to avoid alkylation of α- and ε-amino groups of proteins. Colloidal silver staining of electroblotted proteins on membrane supports yields dark yellow to orange protein bands on a white background, with detection limits of 1–5 ng [99]. The staining technique is compatible with peptide digestion and subsequent analysis by mass spectrometry.

Silver staining of polyacrylamide gels prohibits their subsequent transfer to membranes for Western blotting. Though a double labeling procedure combining colloidal gold for total protein detection and chemiluminescence for immunological detection of specific proteins electroblotted to PVDF membranes has been reported, compatibility with Edman-based and mass spectroscopic analysis has not been determined [108]. Whether colloidal silver staining of nitrocellulose membranes can be combined with immunodetection remains to be determined as well [99].

4.3. Fluorescent detection of proteins for sequencing and immunoblotting

Fluorescent detection of proteins after gel electrophoresis is gaining popularity, particularly with

laboratories engaging in large scale proteome research. Conventional fluorescent labels require covalent modification of free amino, carboxyl or sulfhydryl groups [109–111]. Sensitivity varies considerably from protein to protein depending upon the number of functional groups accessible to modification by the fluorophore. When performing 2D gel electrophoresis or IEF, pre-derivatization with fluorescent molecules may lead to aberrant protein migration [110,111]. Addition of the fluorophore may also decrease protein solubility [111]. This can be compensated for by labeling a small percentage of the functional groups in a sample but slight molecular mass differences between labeled and unlabeled protein could lead to errors in post-separation protein identification by Edman-degradation or mass spectroscopy.

Non-covalent fluorescent protein stains, such as SYPRO Red dye, SYPRO Orange dye and bathophenanthroline disulfonate-europium (BPSA-Eu) can be utilized in conjunction with differential detergent fractionation and computerized image analysis to rapidly identify protein subcellular redistribution events (Fig. 4). SYPRO Red and SYPRO Orange stain (Molecular Probes, Eugene, OR, USA) are best suited for visualization of proteins in polyacrylamide gels, though membrane supports can be stained with approximately 25% of the sensitivity achieved in gels [112–117]. SYPRO Red and SYPRO Orange dyes can detect proteins with a simple, one-step staining procedure using 7% acetic acid in water, and no subsequent destaining step is needed. In 1D polyacrylamide gels the procedure only requires 45 to 60 min to complete, while best results are achieved for 2D gels by staining for 3–5 h. As little as 1 to 10 ng of protein can be detected, rivaling the sensitivity of standard silver staining techniques. Since staining is due to intercalation of dye in the SDS micelle, little protein to protein variability is observed in SDS–PAGE compared with amine-directed stains such as CBB. Consequently, certain proteins that stain poorly with CBB such as phospholamban are readily detected by SYPRO dye staining [118]. Documentation of stained gels can readily be achieved by photography on a standard laboratory UV transilluminator. Alternatively, the dyes can be quantified with commercially available CCD camera-based image analysis workstations or

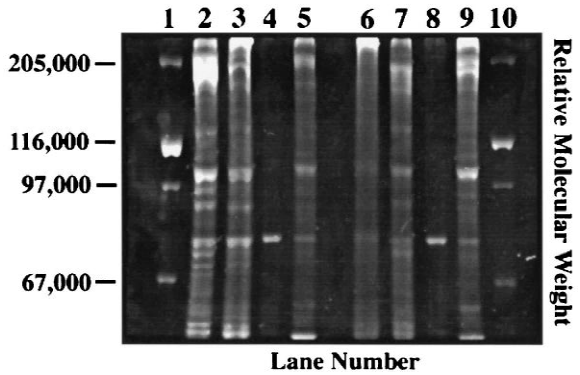

Fig. 4. Myosin redistribution in hydrogen peroxide-treated retinal pericytes. Myosin, like filamin is poorly separated by current 2D methods. Control and hydrogen peroxide treated pericytes are fractionated by sequential, differential detergent fractionation [86]. Protein fractions are resolved on an 8% SDS–polyacrylamide gel and stained utilizing SYPRO Orange dye (Molecular Probes, Eugene, OR, USA). Lanes 1 and 10; molecular mass standards, lanes 2–5; cytosol, membrane/organelle, nuclear and cytoskeletal fractions of untreated cells, lanes 6–9; cytosol, membrane/organelle, nuclear and cytoskeletal fractions of hydrogen peroxide treated cells. Two hundred and fifty μM hydrogen peroxide causes a redistribution of myosin from the cytosol to the cytoskeletal fraction (note doublet running slightly below the M_r 205 000 standard). Photograph courtesy of Dr. Negin Shojaee of Boston University and Ms. Kiera Berrgren, Molecular Probes, Eugene, Oregon.

laser scanners, providing a linear dynamic range of 2.5- to 3-orders of magnitude. SYPRO Red stain is spectrally well matched to 532 nm and 633 nm lasers while SYPRO Orange stain is well suited for 450, 473 and 488 nm lasers. After staining, proteins can also be transferred to membranes by electroblotting and specific proteins identified by immunological staining, Edman-based sequencing or mass spectrometry [114,116,117]. SYPRO dyes are some of the few stains that are capable of detecting proteins in CE as well as in gels and on electroblots, providing a high level of flexibility for proteome studies.

BPSA-Eu can be used as a readily reversible protein stain on nitrocellulose and PVDF membranes [119–121]. Similar to the SYPRO dyes, BPSA-Eu is visualized on a standard UV light box at 300 nm, with sharp emission maxima at 590 and 615 nm. Since binding is mediated by dye sulfonate interaction with protein primary amines, more protein-to-protein variability is expected using BPSA-Eu com-

pared with the SYPRO dyes. Blots are stained at acidic pH and dye can be eluted by incubating in phosphate-buffered saline at pH 7.0. Alternatively, since the stain is only visible upon illumination with a UV light source, colorimetric or chemiluminescent immunodetection can be performed without destaining. BPSA-Eu exhibits a 500-fold linear dynamic range, and detection sensitivity of less than 1 ng/mm^2 of membrane surface as determined using a CCD-camera based instrument [119]. Luminescent detection of proteins in polyacrylamide gels with BPSA-Eu requires destaining just like CBB but provides detection sensitivity comparable to silver staining. BPSA-Eu does not modify proteins, and is compatible with immunoblotting, mass spectrometry [matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)] and Edman-based protein sequencing [119–121].

Changes in the subcellular distribution of proteins are readily determined using SYPRO Red dye, SYPRO Orange dye or BPSA-Eu in conjunction with differential detergent fractionation. Detergent fractions may be resolved by 1D or 2D gel electrophoresis or after electroblotting to nitrocellulose membrane. For laboratories that perform sequencing within gels, SYPRO Orange or SYPRO Red stain can be used to detect proteins without the necessity for a destaining step. Laboratories that perform sequencing on membrane supports can use BPSA-Eu for high sensitivity protein detection. High-throughput analysis of a protein of interest can be performed by vacuum slot-blotting detergent fractions without electrophoresis to nitrocellulose membranes as arrays [119]. Total protein is determined with BPSA-Eu stain and membranes are then re-probed for specific proteins using conventional immunoblotting techniques.

4.4. Image analysis and databasing

Though most stains are readily visualized by eye, a computerized CCD-camera- or laser scanner-based image analysis system should be utilized for data acquisition to realize the full potential of gel-based protein translocation technology, since quantitative information can be obtained using image processing and analysis software [47,122]. The majority of our analysis is performed on a BioImage workstation from Genomic Solutions, Ann Arbor, MI, USA. Images are obtained by digitizing at about 1024× 1024 picture elements (pixels) resolution with 8-, 12-, or 16-bit gray scale levels per pixel [47,122]. A 0.3 absorbance unit neutral density filter and a 600±70 nm band pass filter are used for image analysis of CBB stained gels, a 450±70 nm band pass filter is used for silver stained gels, and autoradiographs simply require a neutral density filter. A low light CCD-camera system allows integration of fluorescent signals for a minute or more and thus increases detection sensitivity. Gels or membranes are illuminated using a standard laboratory UV light box. SYPRO Red dye, SYPRO Orange dye, and BPSA-Eu stained gels are best visualized using a 490 nm long pass filter such as Kodak Wratten gelatin filter No. 9 (S-6656; Molecular Probes) [115]. With the BPSA-Eu, pulsed excitation light from a xenon flash lamp can be utilized instead of a UV light box to minimize background fluorescence. Most high-end image analysis workstations are capable of comparing multiple gels containing as many as 10 000 spots each. Information about total protein and target protein abundance (i.e., translocating species) is readily interrogated using the database software, while further analysis and tracking of information is readily performed by exporting data files to other programs such as Microsoft Excel (Microsoft Corporation, Bellevue, WA, USA).

4.5. Future potential for automation

The foundation of the proteome strategy delineated in this review article is that many signal transduction events are accompanied by subcellular redistribution of key regulatory proteins (e.g., kinases, cytoskeletal proteins, G-proteins). These compartment-specific changes in protein dynamics are known to be of significant regulatory significance. The selected method of differential detergent extraction fractionates cells into cytosolic, membrane-organelle, nuclear/residual organelle and cytoskeletal fractions [64,68,86]. Since the differential detergent fractionation procedure involves serial extraction of cell monolayers, automation should be relatively simple. Media containing the test agent of interest (e.g., drug, hormone, environmental toxin) is pumped onto cell monolayers for defined periods of

time, and then pumped into a waste reservoir. Next, differential detergent fractionation is performed by serially pumping each reagent onto the cell monolayer and subsequently pumping the resulting detergent extract into a dispensing reservoir for subsequent apportionment onto the detection array (microplate or membrane).

The essential features of a technology for automatically fractionating cells is already embodied in gel processing instruments such as the Hoefer Automated Gel Stainer from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Though never commercialized, working prototypes of a similar device were designed and manufactured at Millipore Corporation in 1990 (Bedford, MA, USA). In this instrument a pump is responsible for propelling reagents, while valved manifolds serve to regulate the passage of reagents through the system. During the running of a gel processing protocol, programmed instructions electronically trigger the appropriate stainless steel valves to open and close and the pump to transport the required volume of reagents from the bottles to the gel boxes and back. The rocker, located on top of the gel processor cabinet, is equipped with manifolds that direct the passage of reagents into and out of the gel staining boxes. A cell treatment/fractionation instrument is envisioned to utilize the same basic components as the gel processor. The rocker is expected to hold cultured cells instead of gels, however. Reagents are delivered individually to each cell monolayer and subcellular fractions could ultimately be dispensed as arrays onto a nitrocellulose or PVDF membrane or into 96-well microtiter filtration plates. Automation of a protein labeling module for the membrane arrays could be based upon similar design criteria as the automated silver staining unit described.

5. Utilizing proteomics to screen pathways impinging upon a protein target

5.1. Screening proteins with pharmacological activators and inhibitors

Numerous signal transduction pathways often impinge upon a single protein target, such as filamin. For example, bradykinin is well known to activate a broad range of second messenger pathways including CaM-PK II, protein kinase C, tyrosine kinases, tyrosine phosphatases, serine/threonine phosphatases, MAP kinase and phospholipase D [123,124]. It is thus often necessary to screen a large number of second messenger pathways in order to ascertain which pathway is critical to the translocation of a particular protein target.

In a target identification program, specific and effective non-peptide pharmacological inhibitors and activators of protein kinases such as ML-7, KN-62 and calphostin C can be enlisted to evaluate protein redistribution events between subcellular compartments [64–70,125]. Inactive analogs for many of the agents are commercially available to serve as controls and redundancy can be built into screening programs so that multiple activators or inhibitors of a pathway must give similar results before a particular pathway is implicated [70].

In such a scenario, subcellular fractions are deposited in an array onto a nitrocellulose support without the need for electrophoresis. Membrane arrays should first be probed for total protein using a reversible stain such as a SYPRO dye or BPSA-Eu for identification of agents that lead to non-specific redistribution of numerous proteins [95]. As an example, cytochalasins depolymerize the microfilament network of cells, leading to the release of a variety of cytoskeletal proteins from the cytoskeletal. This is readily determined utilizing the total protein probes and agents with this property can be flagged. Membranes are subsequently probed for the target protein of interest by standard immunological methods. Subcellular redistribution events are determined with the aid of computerized image analysis workstations. Chromogenic detection of antibodies can be performed to generate a permanent record of target protein distribution. Alternatively, fluorescent or chemiluminescent detection methods can be employed.

5.2. Exploiting protein consensus sequence motifs

Ultimately, batteries of membrane permeant, synthetic peptides may provide powerful tools for probing signal transduction on the proteome scale [65,70,72,124,126]. In particular, modulators of specific protein–protein or protein–substrate interactions

Table 2
Examples of short peptide sequence motifs that have been successfully employed to dissect signal transduction pathways in mammalian cells

Peptide	Target	Peptide sequence (Ref.)
Antamanide	Potassium channel blocker	VPPAFFPPFF (cyclic) ([67])
CaM peptide	Filamin's CaM-PK II site	TGPRLVSNHSLHE ([65])
PIP_2 peptide	Cofilin's PIP_2-binding site	GWAPECAPLKSKM ([70,138])
Camstatin	Calmodulin antagonist	APETERAAVAIQAQFRKFQKKKAGS ([129])
SM-1 peptide	Myosin's MLCK site	AKKLSKDRMKKYMARRXWQKTG ([127])
Acto-myosin peptide	Myosin's actin binding site	IRICRKG ([128])

Amino acids are represented by their standard one letter designation and sequences are displayed with N-terminal to C-terminal orientation. Often an N-terminal acetyl and C-terminal amide is utilized for sequences corresponding to internal protein fragments, and a myristoyl group is appended to the peptides to facilitate cell penetration [65,70].

such as isozyme-selective translocation activators and inhibitors and isozyme-specific competitive inhibitors of protein kinases and phosphatases, would greatly facilitate signal transduction studies [72,127–132] (Table 2).

Guidance for the development of such reagents is obtained by detailed analysis of consensus sequence motifs in various proteins and further refined by screening degenerate peptides to optimize substrate specificity [132–134]. The calcium–calmodulin-dependent protein kinase (CaM-PK) family is used to illustrate the process. Pharmacological agents like KN-62 and KN-93 inhibit the CaM-PK II multigene family as well as CaM-PK IV. Since peptide-based protein kinase inhibitors are likely to better mimic protein–protein or protein–substrate binding sites, they offer the potential of greater specificity compared to the cited pharmacological inhibitors. For instance, though substrate recognition by CaM-PK II and IV are overlapping, the realization that an amino-terminal phosphorylation site on synapsin I is readily phosphorylated by CaM-PK IV but not CaM-PK IIα allowed systematic evaluation of amino acid context requirements and ultimate elucidation of more sophisticated consensus sequences that differentiate between the two kinases [132,133,135,136].

We utilized consensus sequence information to develop an inhibitor of filamin translocation based upon the CaM-PK II pathway [64–66]. Since the glycoprotein binding domain of filamin is located near the C-terminus of the protein, the protein sequence was searched for consensus CaM-PK II phosphorylation sites in this vicinity (Table 2) [65]. A myristoylated synthetic peptide derived from this region blocks bradykinin-induced translocation of filamin in cultured endothelial cells [65]. In a similar strategy, we attempted to block calcium-independent redistribution of filamin induced by hydrogen peroxide [68–70]. Increased synthesis of phosphatidylinositol 4,5-biphosphate (PIP_2) accompanies filamin translocation [70]. Though PIP_2 binds filamin, causing it to disassociate from actin, the specific binding site has not yet been mapped [137]. The PIP_2 binding site of another cytoskeletal protein, cofilin, has been determined and this sequence was utilized to generate a myristoylated synthetic peptide that prevents hydrogen peroxide-induced filamin subcellular redistribution [70,138] (Table 2).

5.3. Validating protein translocation events

An overall objective of biology is to elucidate molecular mechanisms responsible for human disease so as to aid in developing strategies for prophylactic and therapeutic intervention. The physiological ramifications of molecular alterations such as protein subcellular redistribution should be established in the context of disease processes. The redistribution of a protein such as filamin in response to a vasoactive agonist is at best a surrogate marker for the changes leading to edema and swelling that arise from inflammation: A key issue is whether blood vessel permeability can be prevented by blocking filamin.

Once molecular targets have been identified and key second messenger pathways impinging upon the targets defined, other screens more closely affiliated with the physiological phenomenon under study should be considered. The two myristoylated peptides that prevent bradykinin- and hydrogen perox-

ide-induced filamin translocation (CaM and PIP_2 peptides in Table 2) were subsequently evaluated in a variety of other tissue culture assays of inflammation [64,70]. Inhibiting filamin translocation was found to also prevent F-actin rearrangement, intercellular gap formation, cell migration and nitric oxide synthase redistribution.

The strength of the proteome approach in uncovering new therapeutic interdictions is especially evident from the cited filamin translocation experiments. Inflammation is a very mature field of study and numerous approaches have been devised for negating the process. Despite the seemingly saturated nature of the discipline, the proteome approach outlined in this review article generated two new drug candidate leads tailored to the prevention of endothelial cell junctional leakage.

6. Conclusions

To date proteome analysis has not dealt with proteins having relative molecular masses ranging from 200 000 (myosin) to 3 000 000 (titin). Focused efforts are needed to develop 2D technology that can simultaneously resolve numerous high-molecular-mass proteins in a single electrophoretic profile. Another important challenge in separation sciences is to be able to measure dynamic protein movements occurring during cell activation. In conjunction with a differential detergent fractionation procedure, and computerized image analysis, luminescent probes such as SYPRO Red dye, SYPRO Orange dye and BPSA-Eu allow rapid mapping of signal transduction pathways by monitoring protein subcellular translocation. For high-molecular-mass proteins, differential detergent fractionation combined with SDS–PAGE or membrane arrays is often adequate for examining signal transduction cascades that lead to protein subcellular redistribution. Quantitative analysis of protein redistribution readily augments morphological studies obtained by immunofluorescence microscopy. The quantitative detection of protein translocation complements other protein-based technologies such as those that determine protein post-translational modifications, protein–protein interactions and protein–nucleic acid interactions.

Acknowledgements

The author thanks Qin Wang, Laurie Hastie, Mark Lim, Negin Shojaee, Eddie Chiang, Catherine Lee, Kimberley Spofford, Nancy Chung-Welch and David Kang for assisting in the development of subcellular fractionation and protein detection strategies to analyze signal transduction events in pericytes and endothelial cells.

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