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Review

Functional proteomics; current achievements

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Abstract

This review presents the current improvements in functional proteomic strategies and their research applications. Proteomics has emerged as an indispensable methodology for large-scale and high-throughput protein analyses in the post-genome era. Functional proteomics, the comprehensive analysis of proteins with special attention to their functions, is a powerful and useful approach for investigations in the life and medical sciences. Various methods have been developed for this purpose, expanding the field further. This important technology will not only provide a wealth of information on proteins, but also contribute synergistically to the understanding of life with other systematic technologies such as gene chips. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Proteomics

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1. Introduction

The human genome-sequencing project is one of the most prominent and exciting missions in human science. The genome sequence is already available in draft form and the final sequence is expected to be completed in 2003 [1,2]. These genomic analyses promise to provide much knowledge about the life sciences and biomedical research. The genome sequence contains an enormous amount of information on all the biological mechanisms by which organisms maintain life and adapt to environmental changes. However, we are far from the ultimate goal of understanding life completely, because we do not possess the tools with which to elucidate all biological mechanisms solely from genomes. Gene prediction from the genome sequence is often insufficient to identify all genes at present because the evidence on which prediction algorithms are based is still inadequate [3,4]. Comparative expression analyses of mRNAs and proteins have shown that expression levels of mRNAs are not necessarily correlated with those of the encoded proteins [5,6]. In addition, there are biological samples such as body fluids with no mRNA but with obvious importance in biomedical research. Therefore, it is necessary for an understanding of dynamic physiological phenomena to analyze systematically their nature and mechanisms of action at the very site of occurrence [7]. ‘‘Proteomics’’, the currently emerging technology leading to large-scale and high-throughput identification and understanding of proteins, serves us an alternative tool to existing analytical methods for describing life in molecular terms [8].

Since the term ‘‘proteome’’ was proposed in 1995 as a concept of the protein complements expressed in a genome, a cell or a tissue, proteomics has developed rapidly as a field to study proteomes [9,10]. Classically, two-dimensional (2-D) gel electrophoresis performed as a combination of isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) had been the only method to analyze the proteome with high resolution [11]. With this method, the thousands of proteins that are expressed in a specific cell or a specific tissue can be separated. Hitherto, no method had been developed with a resolution greater than 2-D gel electrophoresis. To a certain extent this high resolution reveals what proteins are working in the cell at a specific state, which represents the protein signature of the cell [12]. In this approach, the differentially expressed proteins in response to changes in cellular states are especially focused and analyzed. This methodology, termed ‘‘differential-display proteomics’’ or ‘‘expression proteomics’’, worked as a major driving force behind proteomic analysis.

Although 2-D electrophoresis is a powerful and important technique for proteomic analysis, it still has methodological limitations, i.e. it does not have sufficient resolution and dynamic range to separate all cellular proteins in a single operation. The 2-D method does not have a high enough resolution to separate multiple species originating from a single protein with post-translational modifications, such as those with carbohydrates [13]. In addition, we often fail to detect proteins on 2-D electrophoresis gels with molecular masses in excess of 100 thousand daltons and those with p*I* values lower than 4 or higher than 9. Moreover, conventional 2-D electrophoresis only shows protein expression and cannot detect protein–protein interactions and protein functions in principle without using particular methods such as affinity electrophoresis [14]. Thus, other approaches are required to enable a comprehensive understanding of cellular mechanisms at the protein level. For this purpose, we need to know how each protein acts and which proteins are functionally interrelated. This emerging field of systematic protein analyses focusing on protein function, its interactions, and biological phenomena is termed ‘‘functional proteomics’’. Many strategies have been de-

Methods used in proteomic

(1) Expression proteomics
- **Identification of total proteins and differentially expressed proteins**
  - **Two-dimensional electrophoresis**
  - **Multi-dimensional chromatography**

(2) Functional proteomics
- **Interaction proteomic**
  - **Biochemical approaches**
    - **Direct analysis of functional protein complexes**
    - **Affinity purification**
    - **Protein chips**
    - **BIA-MS**
  - **Genetic approaches**
    - **Yeast two-hybrid syste**
    - **Phage display**
  - **Bioinformatic approache**
    - **Prediction algorithms**
- **Posttranslational modifications**
  - **Phosphoproteomics**
  - **Glycoproteomics**
  - **Others**
- **Quantitative proteomics**
  - **Differential staining**
  - **Isotope coded affinity tags (ICAT)**

Fig. 1. Categories of proteomic methods.

veloped for functional proteomics and are currently achieving considerable success (Fig. 1). This review presents a survey of recent developments in functional proteomic approaches and investigations using them, and will focus on methodology and its research applications.

2. Progress in technology for proteomics

The draft sequence data of the human genome predicts that the human genome contains around 30 000–40 000 genes, 10 000 of which can be expressed in one cell [8]. Because a large proportion of these proteins is post-translationally modified, the number of protein species in a human cell is even higher than this figure. For the simultaneous analysis of such a large number of proteins, a high-throughput method is indispensable. For this purpose, more efficient proteomic approaches based on mass spectrometry and informatics have been developed and put into practical use in the last decade.

2.1. Mass spectrometry

Much of the recent advance in proteomics is owed to progress in the mass spectrometry (MS) instrument. In the past, protein identification methods, which were represented by Edman degradation and amino acid composition analysis, had been tedious and time-consuming. Even though partial amino acid sequences of proteins predicted from cDNA sequences provide enough information with which to identify the proteins, this method has limitations with respect to rapid and sensitive analysis. The introduction of mass spectrometric methods drastically reinforced protein identification strategies. Especially, the development of the time-of-flight (TOF) mass

spectrometer and soft ionization methods, including matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), allowed us to accurately measure the molecular masses of biomolecules. Since the features of the MS apparatus are only described briefly in this review, the reader is referred to other papers describing this field in more detail [15–17].

The first method of choice in protein identification by MS is the peptide mass fingerprinting (PMF) method. In this method, proteins are typically separated by gel electrophoresis, digested with a sequence-specific endopeptidase such as trypsin, and subsequently analyzed by MS. Comparisons of the obtained molecular masses with theoretical values of protein masses registered in peptide mass databases yield identified proteins listed according to the statistical accuracy of correspondence between the experimental and theoretical masses of the peptides. The PMF method is a rapid way of identifying proteins of organisms with completely sequenced genomes. A MALDI-TOF mass spectrometer is suitable for this method because of its high-throughput performance.

An alternative method for protein identification using MS and database searching is tandem MS analysis. For this analysis, either a quadrupole time-of-flight mass spectrometer or an ion-trapping apparatus is commonly used. In both instruments, peptide fragmentation is induced in a collision cell and the molecular masses of the resultant fragment ions are measured. The obtained mass data of the fragment ions are compared with the theoretical masses of fragments from each protein sequence in the databases. This method is an accurate way to identify proteins and is applicable to short sequence data such as those of expression sequence tags. Although tandem de novo sequencing is possible using MS data, it requires a large amount of peptide sample and does not yield accurate results easily.

In combination with the liquid chromatography system, tandem MS allows high-throughput analysis of peptide mixtures [18,19]. The multi-dimensional chromatographic system, especially effective for separation of extremely complex peptide mixtures [20], is also applicable in high-throughput tandem MS analysis [21,22]. This technique makes it possible to analyze peptide mixtures obtained by the protease digestion of many proteins from biological complexes, organelles, and total cells.

2.2. Informatics

Many protein and cDNA sequence data have been deposited in public databases and genome-sequencing data have also been accumulating rapidly in recent years. Bioinformatics is a methodology by which necessary and useful information is extracted from these huge sequence databases. For example, we need to obtain the sequences of the genes encoding specific proteins from a large number of nucleotide sequence data stored in public databases and annotate them. Bioinformatics often helps us to sort accumulated data and obtain characteristics of gene products.

Classically, tools of information analysis such as sequence alignment, homology search, and sequence motif search have been the main pillars of the bioinformatics field. By linking amino acid sequence data with experimental data of secondary and three-dimensional structures of proteins, the field of protein structure prediction is expanding. In addition, many algorithms have been developed to deduce protein structures and functions from genome sequence data of various organisms (Section 3.3).

Alongside the progress in the mass spectrometry of proteins and peptides, appropriate analytical software that directly identifies proteins consistent with mass spectrometric data has been developed. Although some tools are commercially available, several are also available from public World Wide Web sites such as PROWL [23], Protein prospector [24,25], PeptideSearch [26], Sherpa [27], and Mascot [28]. These programs constitute a part of the currently developing informatics.

2.3. Technology is driving functional proteomics

The improvement in technology mentioned above allows us to perform systematic protein analysis with high throughput and sensitivity. Comprehensive approaches are about to replace the analytical methods of the previous era. The overwhelmingly large quantity of analytical output has the potential to change the strategies of biological research. For example, it may become possible to explain bio-

logical pathways of interest through identification of all the proteins involved. We thus describe below how effective systematic approaches are in investigating protein functions.

3. Interaction proteomics

When functioning, proteins rarely act as single isolated proteins. In general, functionally related proteins interact with each other. Conversely, one possible approach to elucidate the function(s) of an unknown protein is to investigate the functions of proteins interacting with it. The systematic investigation of protein interactions for the purpose of elucidating protein functions is termed ''interaction proteomics''. There are several approaches to analyze protein interactions, based on (1) biochemical analysis of multi-protein complexes, for example pull-down and affinity capture methods, (2) molecular biology, e.g. the yeast two-hybrid method, and (3) computational prediction.

3.1. Interaction proteomics based on biochemical analysis

3.1.1. Comprehensive analysis of protein targets

For the understanding of protein function(s), it is effective to systematically analyze its target proteins and deduce the function(s) from the common characteristics of the target proteins. It is based on the idea that targets of a protein with a particular function must have similar features. Based on this notion, a comprehensive analysis of the target proteins of *Escherichia coli* chaperonin protein GroEL was carried out [29]. It was revealed that GroEL interacts with 300 newly synthesized polypeptides and that these polypeptides tend to consist of two or three common structural domains. Thus, comprehensive analysis has proven useful in understanding the mechanisms of protein functions.

In recent years, microarray technologies have been applied to proteomics as well as to genomics [30]. Protein chip technology provides a promising way to assay protein interactions systematically [31,32]. Using microarrays of yeast proteome, it was clearly shown that proteome chips provide the capability for high-throughput screening of molecular interactions [33]. 5800 open reading frames (ORFs) of yeast were cloned and each protein corresponding to the ORFs was highly expressed, purified, and immobilized onto glass slides to form a protein microarray. Screening of calmodulin- and phospholipid-interacting proteins led to the discovery of many new interactions. Using the same platform, kinase substrates were also successfully screened [34]. After incubation with substrate candidates on protein chips onto which 119 yeast kinases were printed, the incorporation of radioactive phosphate into classified kinases was detected. Proteome chips are thus applicable not only to protein interaction analysis but also to screening of protein enzyme activity.

For the full realization of the high-throughput analytical potential of protein chips, there is a hurdle to be overcome: the whole proteome must be properly prepared so as to preserve the usability in interaction analysis. Because the results of screening depend on the quality of each protein, the proteome prepared for the microarray must be validated. However, it is actually impracticable to ensure the proper folding, preservation of activity, and proper post-translational modifications of each protein. In order to ensure quality control, proteins should be expressed in a biological environment similar to that of their native conditions [33]. Moreover, different proteins on chips may require different conditions and additional factors for proper activity. Despite the many problems concerning the quality control of expressed proteins, proteome chips are evidently useful in screening protein interactions in principle.

3.1.2. Analysis of intact multi-protein complexes

Direct proteomic analysis of multi-protein complexes, which consists of a combination of traditional biochemistry and the innovative proteomics methodology, is one of the most effective approaches for investigating protein interactions. The biochemical approaches followed by proteomics analyses are applicable not only to the identification of one-to-one protein interactions but also to the analysis of large complexes consisting of many protein components. Ideally, the simple identification of whole constituents of a protein complex purified from bio-

logical samples on the basis of its biological activity is the best way for the comprehensive investigation of the protein components involved in a particular biological function. There are many reports on proteomic analyses of biological complexes: spliceosome [35–37], the nuclear pore complex and its subcomplex [38,39], the yeast spindle pole [40], *Escherichia coli* ribosome [41], yeast ribosome [21,42], the *Mre11/RAD50* double-strand break repair protein complex [43], transcriptional cofactors (the yeast SAGA complex [44], the human DRIP complex [45], the human ARC (activator-recruited cofactor) complex [46], the human CRSP complex [47]), the anaphase-promoting complex [48–50], human BRCA1-associated proteins [51], and the NMDA (*N*-methyl-D-aspartate) receptor-adhesion protein signaling complex [52]. Also, proteomic analysis of enriched fractions has proven useful in investigations of post-synaptic density [53] and phagosomes [54], among others. These examples illustrate that this approach is suitable for the identification of the proteins involved in known biological functions.

Among the studies using functional proteomic analysis, transcriptional cofactors are most frequently investigated. They revealed that these cofactor complexes contain shared subunits and that different cofactor complexes involved in different transcription pathways have different combinations of constituents and naturally have distinct activities [47]. This example shows that proteomic approaches can explain the function and specificity of biological complexes in terms of their molecular architecture.

In investigations of the nuclear pore complex (NPC), which spans pores in the nuclear envelope and mediates molecular traffic between the nucleus and the cytoplasm, functional proteomics analysis identified the protein constituents from the intact complex enriched from the yeast nuclear fraction. Moreover, electron microscopy visualized the localization of each component on the NPC and elucidated the molecular architecture and transport mechanism of the complex [38]. Besides confirming the existence of the identified proteins in the NPC, this research led to the clarification of the function of each protein in the complex. Therefore, it is important for functional proteomic approaches to feedback protein identification to biological functions.

3.1.3. Analysis of multi-protein complexes with epitope-tagged proteins

If the biological properties of a target protein are unknown, activity-based purification is impossible to undertake. In that case, identification of proteins co-purified with the target protein is a common strategy to adopt in inferring the function of the target protein. Conversely, if the biological functions of target proteins are known, we can deduce the involvement of proteins co-purified with the target proteins in the same biological pathways. In either case, the co-purification approach is effective in the identification of protein functions. Although immunoprecipitation by use of an antibody against a target protein is conventionally chosen, we often encounter difficulty because some immunoprecipitates inevitably contain many proteins non-specifically bound to the target protein due to the lack of specific extraction procedures. For successful proteomic analysis of protein complexes, it is essential to prepare samples with the highest purity and the lowest contamination practicable.

In order to purify protein complexes as efficiently as possible, genetically designed epitope-tagged proteins are often used. Immunoprecipitation of protein complexes with an anti-epitope tag antibody from cells in which the epitope-tagged target protein is expressed is a widely followed method. For example, the PCAF histone acetylase complex [55] and the yeast chromatin-remodeling complex [56] were analyzed from cell preparations using immunoprecipitation of FLAG epitope-tagged proteins expressed in cells. The FLAG epitope peptide has the amino acid sequence DYKDDDDK and is a short peptide that leaves the structures and functions of tagged proteins mostly unchanged. Besides the FLAG epitope, the Myc (EQKLISEEDL) [57,58], HA (YPYDVPDYA) [59] and VSV-G (YTDIEMNRLGK) tags have been reported to produce good results.

Although a protein complex can be purified by the epitope-tagging technique, several contaminating proteins usually exist in prepared samples. With the aim of decreasing non-specific binding, the tandem (two-step) affinity tag purification (TAP) system was developed and was put to use with considerable success [60]. The TAP tag encodes a calmodulin-binding peptide, a TEV protease-specific cleavage site, and the IgG binding domain of Protein A. A

TAP-tagged target protein and its interaction partners are purified by the first affinity capture with IgG beads followed by elution with TEV protease and the second affinity capture with calmodulin beads with subsequent native elution with EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid]. This procedure permits the purification of an unknown protein complex bound to a target protein with less contamination than conventional methods and is suitable for the identification of proteins in the complex with the use of mass spectrometry. A similar approach was taken for multiple affinity purification using three tags [61].

Using a FLAG-tagged protein, our group investigated the ribosome biogenesis process [62]. Nucleolin is one of the most abundant nucleolar proteins in exponentially growing eukaryotic cells. The protein is known to interact only transiently with ribosomal RNA and pre-ribosomal particles and to become undetectable in mature cytoplasmic ribosomes. In the investigation the pre-ribosomal complex bound to a FLAG-tagged nucleolin was immunoprecipitated with an anti-FLAG epitope peptide antibody (Fig. 2). After SDS–PAGE separation, each protein band was subjected to proteomic analysis, performed with a combination of in-gel trypsin digestion, MALDI-TOF MS, and PMF. The study identified 40 ribosomal proteins from both the large and small ribosome subunits, and 19 non-ribosomal proteins (Fig. 3). Among the identified non-ribosomal proteins were B23 protein, which is known to be related to ribosome biogenesis, and three RNA-helicases. Since more than 10 RNA-helicases are involved in ribosome biogenesis, as revealed by yeast genetic analyses, these RNA-helicases identified in the nucleolin-binding complex may function in ribosome biogenesis in manners similar to those of yeast. The epitope tagging approach was also applied to analyses of the yeast pre-ribosomal complexes [63,64]. These works adopted the TAP tagging technique for the purification of the pre-ribosomal particle and succeeded in identifying proteins whose involvement in ribosome biogenesis was previously unknown.

Prior to these studies, purification of the nucleolin-binding pre-ribosomal complex by immunoprecipitation with an anti-nucleolin antibody was reported [65]. Although a multi-protein pre-ribosomal complex was successfully purified in this work, only three protein components were identified from the complex because of the low analytical power of conventional protein chemistry. This example illustrates that the epitope-tagging method followed by proteomic analysis is a powerful approach to identify protein constituents of multi-protein complexes.

3.1.4. Separation methods suitable for proteomic analysis of multi-protein complexes

Although 2-D gel electrophoresis is traditionally adopted for proteomic analyses of total cellular proteins, the method is not always required in the analyses of protein complexes consisting of less than ~100 proteins. Moreover, proteins with highly basic p*I* values or high molecular masses (>100 kDa) are not separated on conventional 2-D electrophoresis. In an experiment conducted in our laboratory, separation of the nucleolin-binding complex by conventional 2-D electrophoresis did not result in separation of most of the ribosomal proteins [62]. It is necessary to select a separation method suitable for the samples to be analyzed, or to try various separation modes.

Most works in proteomic analysis of protein complexes are based on gel electrophoresis coupled with mass spectrometry. However, gel electrophoresis and in-gel digestion are steps to be avoided for sensitive analysis whenever possible, because of the long time required, low recovery from gels, and the potential for contamination during the procedure. Liquid chromatography (LC) equipped with the tandem mass spectrometry analysis system represents one solution to overcome these problems. For example, protein constituents of yeast ribosomes were analyzed and identified by multi-dimensional LC and tandem mass spectrometry (MS/MS) [21]. We also analyzed the nucleolin-binding complex by nano-flow LC–MS/MS (Fig. 2) and ascertained that more proteins are identifiable by nano-flow LC–MS/MS than the gel-based PMF method (unpublished).

3.1.5. Biomolecular interaction analysis–mass spectrometry (BIA–MS)

An interesting biochemical approach coupling biosensor chips with mass spectrometric protein identification has emerged. As this system has the discovery-oriented concept in the sense that both the

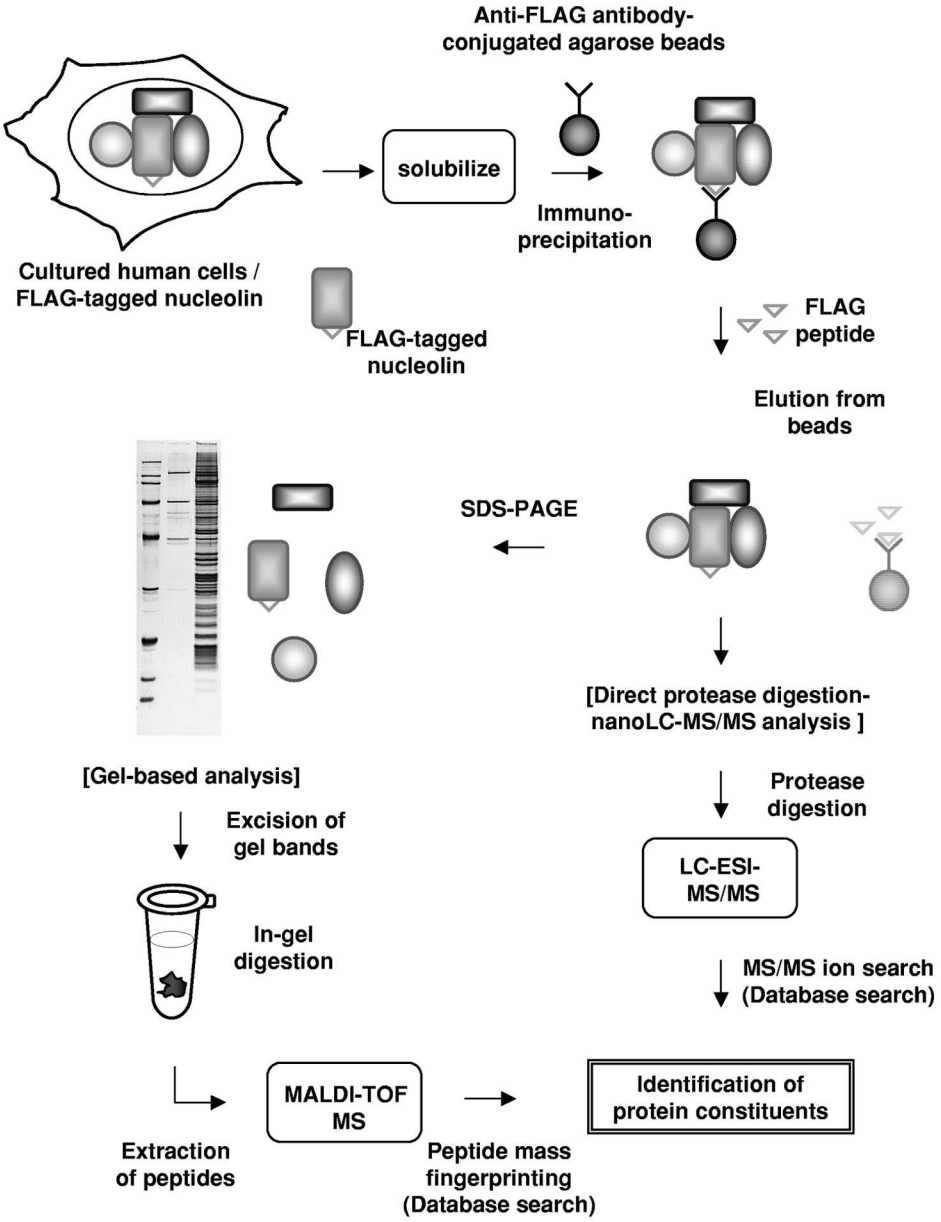

Fig. 2. Scheme of the proteomic analysis of protein complexes using the epitope tag affinity method. An epitope-tagged protein, for which FLAG-tagged nucleolin serves as an example, was transiently expressed in cultured mammalian cells. The nucleolin-binding ribonucleoprotein complex is immunoprecipitated from cellular extracts with agarose beads conjugated with an anti-FLAG antibody and is subsequently eluted. The prepared complex is subjected to SDS–PAGE and the resolved proteins are analyzed individually by MALDI-TOF MS after in-gel digestion to reveal their identities. Alternatively, the prepared nucleolin-binding complex is directly digested with a sequence-specific protease and analyzed by the LC–ESI-MS/MS system.

analysis of interaction partners on biosensor chips and their sequential identification by mass spectrometry are performed in a concerted system, it is called ‘‘biomolecular interaction analysis–mass spectrometry (BIA–MS)’’ [66]. BIA–MS was originally developed as a combination of a biosensor based on

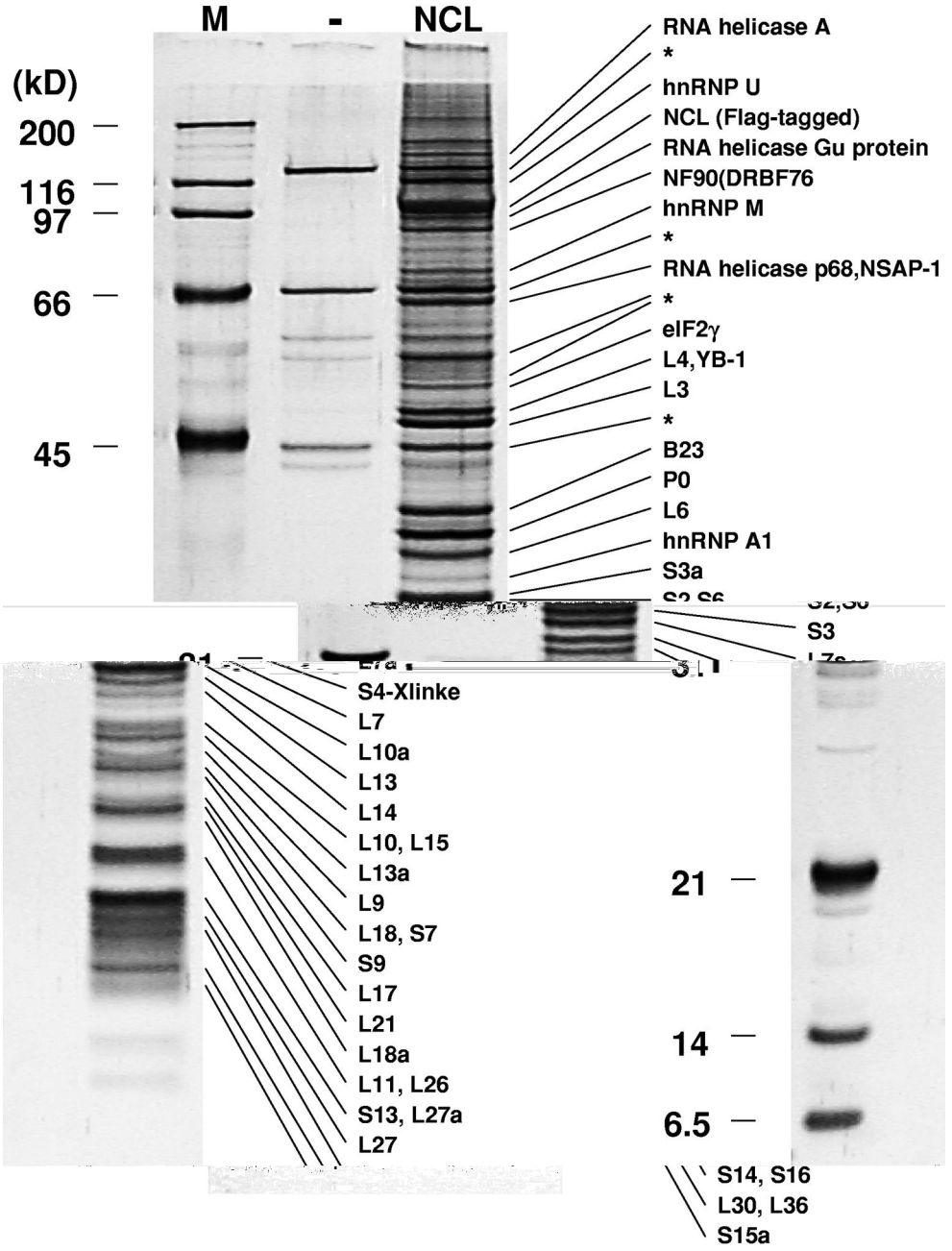

Fig. 3. Identification of proteins co-purified with FLAG-tagged nucleolin. The nucleolin-binding complex was subjected to an 11% SDS–PAGE gel. Abbreviated names of proteins identified by MALDI-TOF MS are indicated on the right. Asterisks indicate the proteins also identified in a negative control experiment [62].

the principle of surface plasmon resonance (SPR) and MALDI-TOF mass spectrometry [67,68]. A SPR biosensor equipped with microfluidics is a useful tool for detecting biomolecular interactions, such as protein–protein, protein–nucleic acid, and receptor–ligand interactions, in real time [69]. The target

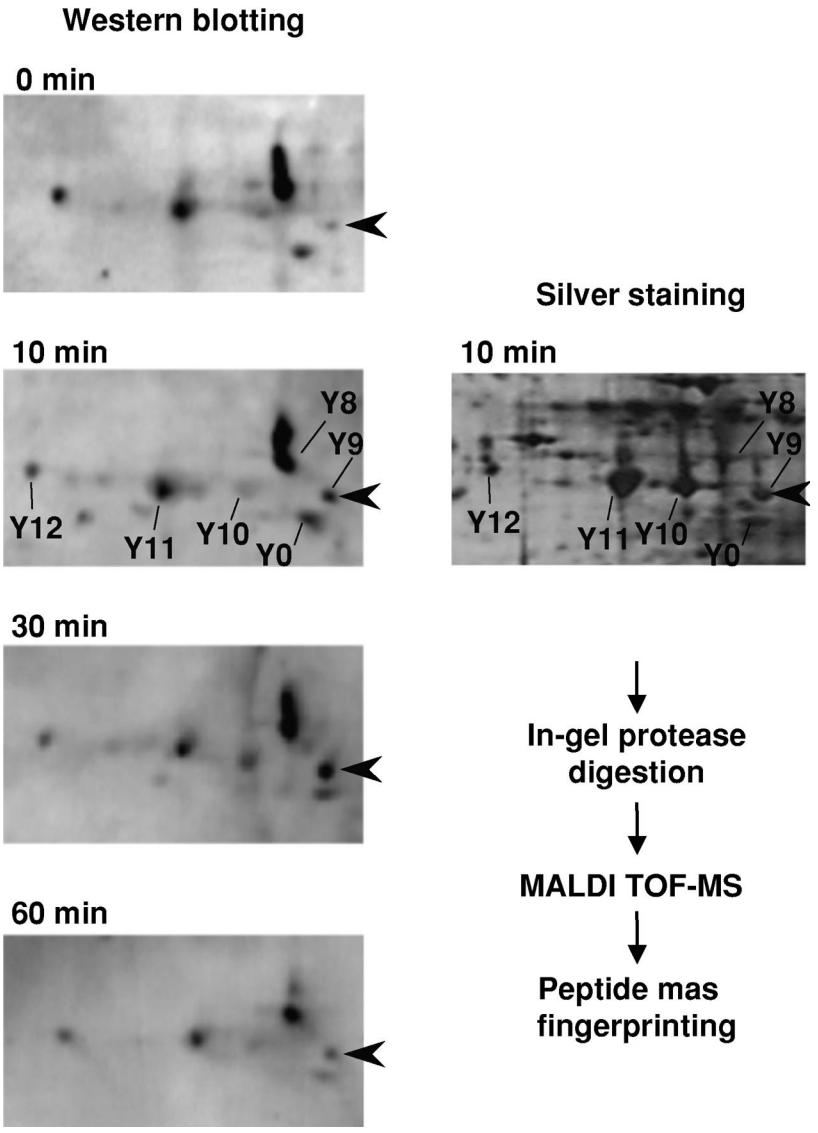

Fig. 4. Identification of Tyr-phosphorylated proteins using a 2-D electrophoresis gel. 2-D electrophoresis of protein extracts from L929 cells 0–60 min after TNF-α stimulation was performed. Subsequently, Western blotting with an anti-phosphotyrosine antibody (left) and silver staining (right) was carried out. The numbered spots on the silver-stained gel corresponding to those on the Western blotting membrane were analyzed by in-gel digestion and MALDI-TOF MS. The spots indicated by arrowheads changed intensity in a time-dependent manner [87].

strenuous efforts for improvement, there remain problems of non-specific binding to non-phosphorylated peptides and insufficient capability to bind all phosphopeptides [95].

Thus, a more efficient method for phosphopeptide enrichment is required as a general analytical technique for phosphopeptides. Phosphopeptide-specific chemical substitutions of the phosphate moieties represent one solution. Basically, two chemical substitution approaches have been reported: one is based on β-elimination of phosphate moieties [96–98], while the other is based on condensation catalyzed by carbodiimide [99]. In the former method, the phosphate moieties of phosphoserine and phos-

phothreonine residues are substituted by a biotin affinity tag and are purified by avidin affinity chromatography. The method is applicable to all phosphoserine and most phosphothreonine residues. By contrast, the latter method introduces the thiol group in place of phosphate and is suitable not only for phosphoserine and phosphothreonine but also for phosphotyrosine. Each method has succeeded in the phosphopeptide enrichment of model proteins and of abundant proteins in biological samples.

Systematic detection of phosphorylated peptides without phosphopeptide enrichment has also been attempted [100]. The employed method scans fragmented molecules derived from phosphate moieties by mass spectrometry. At present, it is hard to judge which approach has the most potential.

4.2. Glycosylation

More than half of all proteins have been estimated to be glycosylated [101], receiving glycosylation either on the hydroxyl group of some serine, threonine, and hydroxylysine residues (O-linked glycosylation) or the amide group of some asparagine residues (N-linked glycosylation). The glycosylation of glycoproteins is required for their proper folding, activity, and stability. The glycosylation patterns of some proteins change according to cellular development stages and in response to diseases. Analysis of glycoprotein, i.e. which proteins are glycosylated and what kinds of sugar chains are added, must give a large amount of valuable information on biological functions. Owing to the heterogeneity in the carbohydrate chains, however, glycosylated proteins are difficult targets of proteomic analyses using gel electrophoresis. Although the number of reports on glycoprotein proteomics is small, hopeful approaches have appeared.

As the 2-D gel electrophoresis analysis of serum proteins containing many glycoproteins has been widely conducted for many years [102–104], basic technical expertise for 2-D gel analysis of glycoproteins has accumulated [105]. The concept of proteome analysis of glycoproteins from 2-D gels and analysis of model proteins has been proposed [106]: after glycoproteins have been separated on 2-D electrophoresis, protein spots are digested, and glycopeptide identification and assignment of the glycosylation site are carried out. In parallel, protein spots are blotted on a PVDF membrane, and monosaccharides and oligosaccharides are analyzed.

Typically, glycoproteins are separated by 2-D gel electrophoresis to ‘‘trains’’ of spots, making them unamenable to 2-D gel analyses [107]. To avoid the difficulty of glycoprotein separation by gel electrophoresis, a non-gel-based strategy has emerged for the analysis of lectin-selected glycopeptides from tryptic digests of a total protein sample [108]. These strategies remain relatively conceptual and have attained limited success in glycoprotein identification because of the complex nature of the samples analyzed. Since glycoproteomics is an important field, it is highly desirable that analytical methods for this field will be improved in the near future.

4.3. Proteomics specific to other modifications

As mentioned earlier, some systematic methods to analyze post-translational modifications such as phosphorylation and glycosylation are beginning to be developed. However, there are various kinds of protein modifications for which no analytical methods have been established. Modifications that are known to occur on common amino acid sequence motifs are predictable. However, analysis of the proteins themselves is necessary for confirmation of the predicted modifications, since it is unclear whether the proteins are truly modified in their native states. Examples of attempts at a comprehensive analysis of modifications include the following: ubiquitination of the protein involved in energy-dependent protein degradation [109]; addition to proteins of farnesyl and geranylgeranyl isoprenoids, which act as a signal of the intracellular trafficking of proteins [110]; and glycosylphosphatidylinositol anchoring involved in protein localization to the detergent-insoluble fraction (lipid rafts) of cellular membranes [111]. Generic analytical methods for all post-translational modifications are expected to be developed so that post-translational modifications of proteins can be analyzed systematically.

5. Quantitative proteomics

Quantitative analysis of each component in protein mixtures is a key to understanding the mechanisms

of biological processes. For instance, quantification of the amounts of cellular proteins that vary in response to a perturbation is one avenue to the elucidation of the biological mechanisms involved in the perturbation. However, conventional mass spectrometry, with all its capabilities of identifying proteins, is unsuitable for quantifying proteins and determining their changes because the ionization efficiencies of different peptides vary. To overcome this problem, comparative quantifying methods are needed.

In gel-based analyses, conventional staining methods with dyes such as Coomassie Brilliant Blue have been widely used for quantification. The differential fluorescent labeling method was recently developed [112]. Prior to 2-D gel electrophoresis, two protein samples to be compared were covalently labeled with different fluorescent dyes and then mixed and subjected to 2-D gel electrophoresis. A comparison of the fluorescent intensities of two spots on a single gel produces an estimation of the ratio of the amounts of the protein in the two samples. This method has the advantage of not comparing data from two gels, as such a comparison frequently results in poor reproducibility.

On the other hand, an LC–MS-based quantitative approach is gaining prominence. In the field of mass spectrometry, comparative quantification is commonly performed by way of derivatization with a pair of stable isotopes distinguishable by mass spectrometry. This stable isotope labeling strategy was also applied to quantitative proteomics with the introduction of the isotope-coded affinity tag (ICAT) reagent [113]. The ICAT reagent is one of the most popular commercially available chemical reagents and is composed of a thiol-reactive iodeacetamide moiety and a biotin tag moiety with a linker chain with eight deuterium or eight hydrogen atoms inserted between the two. Two samples of cysteine-containing proteins were labeled with ICAT tags with different isotopes, mixed, and subjected to protease digestion to yield peptides. The labeled peptides were enriched by the tag affinity method and analyzed by LC–MS/MS. Quantification was carried out by comparing doublet peaks separated by 8 mass units.

With the use of the ICAT technique, a comprehensive analysis of microsomal protein changes by differentiation stimulus was carried out [114]. The abundance ratios of 491 proteins in the microsomal fraction of human myeloid leukemia HL-60 cells with and without differentiation were estimated and analyzed. The procedure produced a protein expression profile changing with differentiation, which can be viewed at a glance. Thus, systematic quantification of cellular proteins can reveal states of cells.

6. Conclusions and perspectives

Various methods of functional proteomics have been presented in this review. The reader can select and apply these tools to elucidate biological processes of interest. As pointed out above, at present many scientists select budding yeast as a model organism for functional proteomic analyses. The preference for this eukaryotic unicellular organism arises mainly from the fact that the whole genome sequence is available and genetic analysis can easily be carried out. This means that gene annotations and confirmation of the functions of gene products is absolutely indispensable for functional proteomics analyses. Compared with yeast, genome annotations of mammalian species, including *Homo sapiens*, remain rather sketchy and must be refined. Scientists using proteomics will obtain fruitful results from public databases and they should reciprocate by communicating their results to databases and enrich the commonly shared knowledge.

Although the emergence of functional proteomics is revolutionary, the field by itself has insufficient capability for the full elucidation of biological processes. In order to understand biological systems it is beneficial to combine data obtained by functional proteomic analyses with those provided by other systematic methods, such as genomics. For example, proteomic data of the yeast proteins involved in galactose utilization were interpreted in relation to results of gene transcription and known protein interactions, leading to a clear comprehension of the relationship among the components of galactose utilization as a biological system [115]. Thus it will become more and more important for the understanding of biological pathways to evaluate results from functional proteomics with feedback to biological phenomena. If properly developed, functional proteomics will combine all the protein information that has accumulated in the pre-proteomics era with knowledge that will accrue in the next

generation to produce a huge network. This network will deepen our understanding of the molecular mechanisms of life, which is our ultimate theme.

7. Nomenclature

2-D	two-dimensional
ESI	electrospray ionization
IMAC	immobilized metal affinity chromatography
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
MS/MS	tandem mass spectrometry
ORF	open reading frame
PMF	peptide mass fingerprinting
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	surface plasmon resonance
TAP	tandem affinity purification

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