Gel electrophoresis for phosphorylated proteins: a brief introduction

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SUMMARY

Protein phosphorylation is a key mechanism that regulates cellular physiological functions such as proliferation, migration, cell cycle progression, apoptosis, and differentiation. Aberrations in kinase activity and subsequent dysregulation of protein phosphorylation occur in the process of carcinogenesis and cancer progression and are considered to be therapeutic targets and biomarkers in oncology. Gel electrophoresis has versatile utility in the study of protein phosphorylation. Phosphorylated proteins can be enriched prior to gel electrophoresis, and the proteins separated by gel electrophoresis are visualized by colorimetric methods or western blotting with antibodies, which specifically detect phosphorylation. Phosphorylated proteins migrate differently from non-phosphorylated proteins in gels containing substrates with affinity for phosphorylation. All these methods can be combined in multiple ways, generating unique data from different viewpoints. The identification of separated proteins can be achieved by mass spectrometry, making it possible to integrate protein and genetic data. Peptide array allow the evaluation of kinase activity in heterogeneous samples. As protein phosphorylation and kinase activity are under the regulation of multiple mechanisms and their statuses influence the structures and functions of the other proteins, a multidisciplinary approach is required, and gel electrophoresis will play an important role in the study of protein phosphorylation.

Key words: electrophoresis, protein phosphorylation, kinase activity, cancer

INTRODUCTION

Protein phosphorylation is a key mechanism that regulates cellular physiological functions such as proliferation, migration, cell cycle progression, apoptosis, and differentiation. The human genome encodes approximately 500 different kinases that orchestrate these pivotal functions, and 90% of all proteins undergo phosphorylation. Aberrations in kinase activity occur in the process of carcinogenesis and cancer progression due to genetic mutations such as amplification, point mutation, chromosomal translocation, and epigenetic regulation. Moreover, dysregulation of self-phosphorylation and the kinase-to-kinase regulatory relationship is also responsible for the aberrant activity of kinases. Kinases with aberrant activities then disturb important cell functions, transform normal cells to fully malignant tumor cells, and determine malignant features such as invasion, metastasis, and resistance to chemotherapy. Thus, aberrantly regulated kinases and their substrate proteins are considered to be biomarkers that affect the decision-making process in the treatment of cancers. Moreover, protein kinases are major drug targets in oncology, and many kinase inhibitors have been approved for the treatment of cancers. Thus, the study of protein phosphorylation will further our understanding of fundamental biology and lead to novel clinical applications in oncology.

This review aims to depict the application of gel electrophoresis in the study of protein phosphorylation, emphasizing its unique potential.

GEL ELECTROPHORESIS FOR PHOSPHORYLATED PROTEINS

Gel electrophoresis is a powerful method for investigating the status of phosphorylated proteins. In two-dimensional gel electrophoresis, protein phosphorylation is recognized as a shift of protein spots in the isoelectric focusing dimension with multiple protein spots from single genes in the second dimension, which are observed as a result of phosphorylation at multiple sites. We observed that the intensity of multiple protein spots was significantly correlated with clinical observations, and the protein corresponding to these protein spots was identified as a single protein,
Phos-tagTM has flexible utility, which allows its use for phosphorylated proteins, allows the observation of differential phosphorylation among samples by measuring the difference in protein migration during gel electrophoresis. Phos-tag has been previously reported, and we speculated that phosphorylation caused multiple protein spots from the single gene. The intensity of these protein spots synchronously changed, and the total intensity of these protein spots or the total amount of these proteins was a candidate prognostic biomarker in gastrointestinal stromal tumor.

Fluorescent staining methods to detect phosphorylated proteins are also widely used to study the status of protein phosphorylation. Proteins separated by gel electrophoresis are stained with fluorescent dyes, which bind phosphorylated proteins and have different excitation and fluorescent spectra. Using multiple fluorescent dyes with different fluorescent characteristics in identical gels, the proteins separated by a single gel electrophoresis can be characterized and compared between the different gel images. Antibodies specific to phosphorylated proteins also allow the observation of protein phosphorylation in western blotting. Proteins separated by electrophoresis, transferred to a membrane, and reacted with an antibody can be detected in a quantitative way. Owing to the high sensitivity of the detection system, such as enhanced chemiluminescence, a faint amount of phosphorylated protein can be evaluated using western blotting data. Combined use of the fluorescence staining method for the total protein and western blotting using phosphorylated protein-specific antibodies allows for assessment of the degree of phosphorylation, which is standardized by the expression level of subject proteins.

Substrates with an affinity for phosphorylated proteins are implemented in the study of protein phosphorylation. Phos-tag technology, which utilizes molecules with affinity for phosphorylated proteins, allows the observation of differential phosphorylation among samples by measuring the difference in protein migration during gel electrophoresis. Phos-tag has flexible utility, which allows its use to be combined with other methods. For example, kinase phosphorylation is assessed using antibody-specific kinase, namely Multi-PK antibody and Phos-tag 2D-PAGE.

The phosphorylated proteins purified by immobilized metal affinity chromatography (IMAC) or immune-precipitation can also be separated by gel electrophoresis, and the mobility shifts of proteins by phosphorylation are observed on the gel images. Kosako et al. reported on phosphoproteomics in which proteins are purified by IMAC and subjected to analysis by two-dimensional difference gel electrophoresis (2D-DIGE), which allows the comparison of multiple kinases in a single gel. Motani et al. discussed the unique advantages of the combination of IMAC purified proteins, 2D-DIGE, and Phos-tag PAGE. Lind et al. reported the combination of immunoaffinity enrichment, 2D-DIGE, and fluorescent western blotting. The phosphorylated proteins separated by gel electrophoresis can be subjected to mass spectrometric protein identification, and the proteins observed on the gel images can be annotated based on the amino acid sequences. Specific antibodies can also be used for protein identification by western blotting. The variations in experimental procedures for the study of protein phosphorylation are summarized in Fig. 2.

The prediction of protein phosphorylation has been challenged by computational methods, and consensus phosphorylation site motifs for protein kinases have been established. However, such data have been obtained for only about half of all human kinases, and it is poorly understood how kinases achieve specificity for their target substrates. Thus, the data obtained by gel electrophoresis will be useful for validating the results of computational prediction. These previous reports indicate flexible and wide-range utility of gel electrophoresis combined with other multiple methods for the study of phosphorylated proteins.

**ELECTROPHORESIS AND MASS SPECTROMETRY**

Mass spectrometry enables the identification of phosphorylated peptides in a comprehensive way; a single experiment generates the data of phosphorylation of millions of peptides. Large-scale monitoring of kinase-substrate interactions can be achieved in a high-throughput manner. Mass spectrometry has provided enormous amounts of impactful data for cancer research and has an advantage in gel electrophoresis in terms of the intensity of labor. In this approach, sample manipulation can be automatically achieved in multiple steps, and hundreds of samples can be subjected to experiments without manual operations. However, although mass spectrometry allows the analysis of peptides digested by proteases, the overall pictures of differently phosphorylated proteins are difficult to obtain because the proteins are digested to peptides, and they cannot be re-constructed to the original structure.

In contrast with mass spectrometry, gel electrophoresis requires intense labor; although automated systems for gel electrophoresis such as Auto2D BM-100 (Sharp,
Fig. 2 Summary of the experiments for the study of protein phosphorylation.

Fig. 3 Two different approaches to identify phosphorylated proteins using mass spectrometry and gel-electrophoresis, such as 2D-PAGE.
Tokyo, Japan) have been developed, they are not popular in our research community. According to our experience, automated 2D-PAGE is useful for protein purification and evaluation of sample contents\(^{30}\), and its combined use with other methods will provide unique opportunities for the study of phosphorylated proteins. Despite the notorious labor-intensive operation, gel electrophoresis provides information on the full-length protein\(^{30}\), and the different protein isoforms generated by phosphorylation can be distinguished on the gel image (Fig. 3). In contrast with mass spectrometry, the intact full-length proteins are separated and detected in 2D-PAGE. Thus, the integration of the observations about full-length proteins by gel electrophoresis and detailed information about phosphorylated peptides by mass spectrometry will provide exact data on phosphorylated proteins.

**ELECTROPHORESIS FOR KINASE ACTIVITY**

In addition to the phosphorylation of kinase substrate, kinase activity is also important in evaluating the status of molecular backgrounds of cells. In-gel kinase assay is a most famous electrophoretic technique for kinase activity. In the in-gel kinase assay, the protein samples including kinases are separated by SDS-PAGE, in which the gel are polymerized with kinase substrates. After the gel electrophoresis, SDS is washed out and the kinases in the gels are renatured and incubated with \(\gamma\)-\(^{32}\)P ATP. The renatured kinases phosphorylate the proteins in the gel, and detected by autoradiography\(^{31}\). Kameshita et al. reported the unique utility of synthetic peptides which have multiple phosphorylation sites for the detection of a variety of serine and threonine kinases\(^{32}\). They reported the monoclonal antibodies directed to the highly conserved region of protein kinases, and the combined use of such antibodies with the in-gel kinase assay will enable the detection of various serine and threonine kinases with ease\(^{30}\). Bischoff et al. also reported that enzyme activity following gel electrophoresis of a heterogeneous protein mixture is measurable\(^{34}\). Komatsu et al. reported the diced electrophoresis gel assay\(^{35}\) where the proteins are separated by native PAGE with a multiwell-plate-based fluorometric assay. Presently, fluorescent probes for protein phosphorylation are under investigation, and future development is expected.

**TECHNOLOGY OTHER THAN ELECTROPHORESIS FOR KINASE ACTIVITY**

Kinase activity is directly measurable using an in vitro kinase assay. In this assay, the kinase activity is monitored by measuring the transfer of the \(\gamma\)-phosphoryl group from adenosine triphosphate to the hydroxyl group of serine, threonine, and/or tyrosine residue(s) in a protein or polypeptide substrate in the presence of a divalent metal ion\(^{36}\). Based on this principle, global profiling of kinase activity can be performed using a peptide membrane array\(^{37}\). Using more than hundreds of peptides, which are substrates of tyrosine or serine-threonine kinases, on the three-dimensional membrane, a comprehensive kinase activity assay can be performed using heterogeneous protein samples (PamStation, PamGene, HH’s-Hertogenbosch, the Netherlands). The peptide microarray provides data on phosphorylated substrate peptides, and the kinases responsible for phosphorylation are determined by the bioinformatics approach.

**CONCLUSIONS**

Electrophoresis allows the separation of kinases according to their physiological propensities and enables further analysis in combination with multiple other methods. Integration of the data generated by different methods is required to understand the biological meaning of protein phosphorylation. As a fundamental method with versatile utility, electrophoresis will be continuously used for the study of protein phosphorylation.

**ABBREVIATIONS**

IMAC, immobilized metal affinity chromoatography; 2D-DIGE, two-dimensional difference gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PAGE, polyacrylamide gel electrophoresis; ATP, adenosine triphosphate

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