Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 787 (2003) 53–61
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Analysis of state-specific phosphorylation of proteins by two-dimensional gel electrophoresis approach

Hana Kovarova[a,*], Marian Hajduch[b], Mark Livingstone[c], Petr Dzubak[b], Ivan Lefkovits[d]

[a]*Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburska Str. 89, CZ-277 21 Libechov, Czech Republic*
[b]*Laboratory of Experimental Medicine, Department of Pediatrics, Faculty of Medicine, Palacky University, CZ-77515 Olomouc, Czech Republic*
[c]*Cell Signaling Technology, Inc., Beverly, MA, USA*
[d]*Physiology Institute, University of Basel, 4051 Basel, Switzerland*

Abstract

In this paper we focus on the detection of specific state of protein phosphorylation within a complex protein mixture separated by two-dimensional gel electrophoresis followed by immunoblotting. The availability of antibodies that specifically recognize the phosphorylated residue(s) of proteins make this approach feasible as exemplified by the study of the regulatory mechanisms of the cell cycle. The major advantage of the presented approach is its relative simplicity and sensitivity that allows specific detection of protein phosphorylation and distinguishes different phosphorylation sites of target protein. Current findings demonstrate that this method represents a reasonable alternative to the use of other tools to study protein phosphorylation.

Keywords: Protein phosphorylation; Roscovitine

1. Introduction

A variety of post-translational modifications of proteins including phosphorylation, glycosylation, lipidation, acetylation and methylation are important for regulating function, stability, localization, and protein–protein interaction of target proteins. As a consequence, the same protein is often found in several variants which differ upon two-dimensional gel electrophoresis (2-DE) in isoelectric point and molecular mass (Fig. 1). As a rule, the protein variants which were formed by co- or post-translation modifications are encoded by the same transcription unit [1].

Protein phosphorylation is the most studied protein modification by far as it seems that nearly every process in biological systems such as metabolic pathways, cytoskeleton dynamics, signal transduction, gene expression, cell division, apoptosis, etc., is controlled by phosphorylation [2,3]. This observation is in agreement with the estimation that as many as one-third of mammalian gene products can be modified by phosphorylation [4]. Although nine amino acids, e.g., tyrosine, threonine, serine, histidine, glutamic acid, aspartic acid, arginine, cystein, and lysine, can undergo phosphorylation in cells, the

*Corresponding author. Tel.: +420-315-639-564; fax: +420-315-697-186.
E-mail address: kovarova@iapg.cas.cz (H. Kovarova).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00729-8

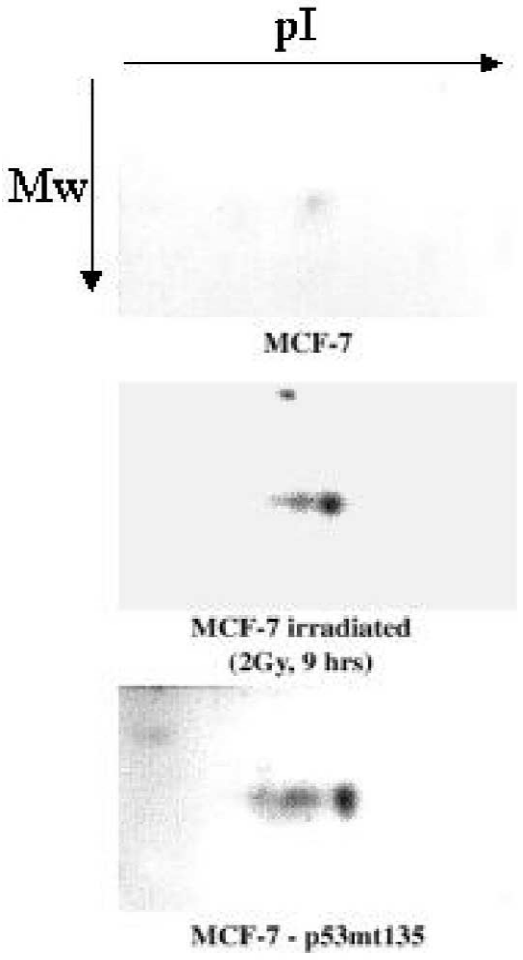

Fig. 1. Separation of p53 protein variants on 2-DE gels. The protein was detected using specific monoclonal anti-p53 antibody (clone BP53-12, Exbio, Prague, Czech Republic). Note enhanced expression and presence of several protein spots of p53 protein in irradiated MCF-7 cells and in cell line stable transfected with dominant-negative mutant (p53mt135, pCMV-p53mt135 vector, Clontech, Palo Alto, CA, USA).

main targets are threonine, serine and tyrosine in eukaryotic cells, while histidine and aspartic acid are preferred in bacteria [5]. The phosphorylation state of proteins is highly dynamic, i.e., it reflects control of biochemical pathways by protein kinases that catalyze phosphorylation, or protein phosphatases that mediate dephosphorylation [6]. In addition, the phosphorylation of a protein by different kinases can occur at distinct amino acid sites [7,8] thus expanding the complexity and protein diversity of the cellular proteome. Since neither the sequence of DNA encoding the protein nor the microarray technology at the transcriptomic level reveals the site(s) and extent of protein modifications, the proteomic approach is of choice to analyze protein phosphorylation and processes related to it.

In this paper we focus on the detection of specific state of protein phosphorylation within a complex protein mixture separated by 2-DE followed by immunoblotting. The availability of antibodies that specifically recognize the phosphorylated residue(s) of proteins make this approach feasible and sensitive, as exemplified by the study of the regulatory mechanism of the cell cycle. Current data demonstrate that the proteomic approach is indeed a suitable alternative to the use of classical techniques such as radiolabelling and immunoblotting by the antibodies that recognize phosphotyrosine, phosphothreonine, phosphoserine.

2. Analysis of protein phosphorylation

The burgeoning field of the proteomics is aimed at profiling and characterizing of gene expression at the translational and posttranslational level. Compared to measurement at the mRNA level, the corresponding proteome provides additional information on taxonomic and functional levels that is directly linked to the observed phenotype [9]. In order to complement known genomes, the classical proteomic approach, e.g., 2-DE coupled with mass spectrometry, have focused on identification of a large number of proteins and quantitative analysis of protein amounts. However, the intrinsic limitations of 2-DE, besides the limited sample capacity and detection sensitivity, are the difficulties related to the presence of multiple protein variants [10]. Although many improvements such as sample prefractionation before 2-DE separation [11], differential sample extraction [12], the use of zoom gels [13], and more sensitive fluorescence dyes [14] have been made, the electrophoresis free high throughput approaches based on array [15] and mass spectrometry (MS) technologies will probably solve technical limitations of 2-DE and become a second-generation proteomics technology in near future [16].

2.1. Identification of phosphoamino peptides by mass spectrometry

A few MS-based methods for identification of phosphorylated proteins have been recently introduced. A first generation technique utilizes enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) that is followed by nanoelectrospray tandem MS analysis allowing identification and sequencing of the phosphopeptides from analyzed proteins [17,18]. However, the major drawback of this method is nonspecific adsorption of nonphosphorylated peptides and low efficiency of adsorption of phosphopeptides in many cases. The second generation introduced derivatization of phosphopetides by fine chemistry before MS analyses to eliminate limits of IMAC method and ''suppressive'' effect of nonphosphorylated peptides. The method of Zhou et al. [19] introduced selective phosphopeptide modification and isolation via formation of phosphoramidate adducts at phosphorylated residues by condensation with cystamine. It seems to be more efficacious in regard to its capability to detect phosphothreonine, phosphoserine, and phosphotyrosine in model proteins, whereas Oda et al. [20] report nonreactivity of phosphotyrosine while using β-elimination of phosphogroup(s) during protein derivatization. In addition β-elimination eliminates the sugars from *O*-glycosylated proteins and results in the same derivatives which must be kept in mind during interpretation of the data.

Although these methods are promising they need optimization of individual steps that would allow the analysis of protein phosphorylation in complex protein mixtures and exploration by multidimensional chromatographic separation for liquid chromatography–tandem MS analyses to detect low abundant phosphopeptides.

2.2. Detection and identification of phosphorylated proteins by 2-DE methods

The most frequent method used in studies on protein phosphorylation involves in vivo or in vitro protein radiolabeling with inorganic ^{32}P or ^{33}P [21]. Following 2-DE separation of labeled proteins and their visualization on gels by autoradiography, the differences in protein phosphorylation among a various phospho-labeled proteomes can be evaluated. In spite of high sensitivity, this method has some drawbacks: (i) it is limited to the cells or tissues which can be labeled, (ii) does not provide access to constitutive physiological state of protein phosphorylation, (iii) the radiation emitted by label induces stress in studied biological system that can interfere with incorporation, (iv) commonly disables the studies of cell cycle, (v) does not identify amino acids which are phosphorylated, and (vi) safety issues with handling radioactive label do not allow high throughput analyses [22].

2.3. Nonradioactive approaches for protein phosphorylation

This situation has prompted effort to improve nonradioactive approaches for studying protein phosphorylation. An alternative to the use of radiolabelling is immunodetection of phosphoproteins separated by 2-DE by antibodies directed against specific phosphorylated amino acid residues. Among available antibodies, the efficacy of antiphosphotyrosine (anti pTyr), namely monoclonal antibody 4G10 (Upstate Biotechnology), has been high [23–25] but still remains rather low for antiphosphothreonine (anti pThr) and antiphosphoserine (anti pSer). The probable explanation of this discrepancy is steric hindrance of too small pThr and pSer residues as the recognition sites in proteins. It appears that these residues can be better recognized in the context of larger epitopes or motifs such as proline in +1 position of phosphorylated threonine, typical for MAPKs (mitogen-activated protein kinase) and CDKs (cyclin-dependent kinase) signaling [26]. The detection of phosphorylated proteins by immunoblotting following 2-DE separation of protein mixture is simple and sensitive. In addition, it can be coupled to identification of the protein by MS analysis. Global strategy is straightforward and consists of these steps [26]:

(i) analyzed sample is separated in parallel on analytical as well as preparative 2-DE gels;

(ii) the analytical gel is used for immunodetection of phosphorylated proteins by immunoblotting with antiphosphoamino acid antibody followed by staining of total proteins by colloidal gold on immunoblots;

(iii) the preparative gel is stained by Coomassie blue;

(iv) matching of immunoblots to preparative gel allows assignment of phosphorylated proteins to their corresponding spots on Coomassie-stained gel;

(v) identification of phosphoproteins is performed by peptide mass fingerprinting.

In general, this approach allows high throughput analysis of phosphorylated proteins including the type of amino acids (tyrosine/threonine/serine) that are phosphorylated. Furthermore, phosphorylated peptides and site of phosphorylation in the identified proteins can be found by searching databases (MS-Fit, Mascot) and NetPhos WWW server (http://www.cbs.dtu.dk/services/NetPhos) providing information on predictive phosphorylation sites in eukaryotic proteins. However, it is evident that intrinsic limitations of 2-DE mentioned above as well as quality of anti-phosphoamino acid antibodies restrict the analysis of complex phosphoproteome. Usually, the low abundance proteins mediating fine regulatory mechanisms and signaling pathways of cellular processes remain hidden in many cases. Therefore, it is still necessary to combine several methods, e.g., electrophoresis free tandem MS analyses and 2-DE-based analyses, to access the pictures of cellular phosphoproteomes and studying the changes under varying conditions.

3. Relevance of protein phosphorylation to disease

An increasing number of diseases ranging from genetic to infectious disease and cancer are becoming known to be associated with perturbation in protein phosphorylation. This reflects the changes in balance between activities of kinases and phosphatases as the result of their mutation, overexpression or inhibition [1,2,27]. Maybe the best realization came from the studies of cell cycle regulation and carcinogenesis. The transformation of a normal human cell to a cancer cell is a multistep process that results in unconstrained cellular proliferation and an aberrant cell cycle regulation. It is known that the precise control of cell cycle is mediated by the fine balanced activity of cyclin-dependent kinases (CDKs). The activity of CDKs is regulated by a complex set of mechanisms, including regulatory phosphorylations and interactions with activating cyclins and the natural CDK inhibitors (CDKIs). CDKs operate at the G1/S and G2/M boundaries, and control the progression through the S phase of the cell cycle [28]. Furthermore, several signaling pathways such as Ras-MAPK and TGF-β are activated through receptor tyrosine kinases and are important for prediction of cancer development and outcome. Studying the phosphorylation of the receptor tyrosine kinase HER2/NEU/ERBB2 and its connection to patient outcome in breast cancer, it was demonstrated that the phosphorylation status of proteins of important signaling pathways relevant to disease provides important prognostic or predictive information [29]. Taken together, these findings indicated the necessity to develop new strategies that provide significant clinical information that is not accessible by other means. The proteomic approach offers the advantage to analyze by a single technique a large number of measurements at the protein level and extract from these data possible clinically relevant information.

3.1. Detection of state of protein phosphorylation by 2-DE coupled to specific immunodetection

Classical proteomic (2-DE×MS) investigation of potential individual markers or groups of markers that have strong prognostic or predictive significance with clinical utility has been usually based on the determination of quantitative differences in protein abundances and identification of protein of interest [30]. Nevertheless, the unraveling of protein function from abundance changes is limited to proteins function of which is not regulated by protein modification and which are represented by single protein spot on 2-DE. In case of phosphorylated proteins, significant regulation of protein function quite often occurs without a change in total protein abundance. Thus, the protein abundance of the hypophosphorylated protein variant can be shifted in a favor of mono-, di-, or pluri-phosphorylated protein variants. Although 2-DE separation is capable of distinguishing phosphorylated variants [31], it does not distinguish which amino acids are actually phosphorylated.

3.2. Strategies for the development and evaluation of phosphorylation state specific antibodies

Phosphospecific polyclonal antibodies directed against known sites of phosphorylation are produced by immunizing animals (generally rabbits, goats, or sheep) with synthetic peptides carrying a phosphate group on the proper amino acid residue. Immunoglobulin is purified using protein A-Sepharose before antibodies reactive with the nonphosphopeptide are removed by adsorption to a nonphosphopeptide affinity column. Antibodies that fail to bind this nonphosphopeptide are next passed over a column of immobilized phosphopeptide, and phosphospecific antibodies are collected by eluting at low pH [32]. Analysis of the phosphospecificity of the resulting antibodies is performed by (i) enzyme-linked immunosorbent assay against the phosphopeptides and nonphosphopeptide, (ii) immunoblotting against whole-cell extracts from cells subjected to a relevant treatment and (iii) immunoblotting using over-expressed or bacterially expressed wild-type and mutant protein (Fig. 2). Mutation to alanine is often used to mimic constitutively nonphosphorylated serine and threonine, while phenylalanine is routinely substituted for tyrosine to prevent phosphorylation [33]. When Western blotting fails to conclusively demonstrate phosphospecificity (e.g., protein is constitutively phosphorylated), broadly reactive phosphatases (lambda or CIP) can be used to dephosphorylate the protein within the cell lysate or directly on the immunoblotting membrane [34]. Also, since nonphosphorylated protein has a distinct p*I* from its phosphorylated form, 2-DE can also be used to assay the phosphospecificity of an antibody [35].

While determining phosphospecificity is a fairly straightforward process, verifying the identity of the major immunoreactive band by immunoblotting is often less conclusive. The interpretation is complicated by the existence of multiple isoforms (e.g.,

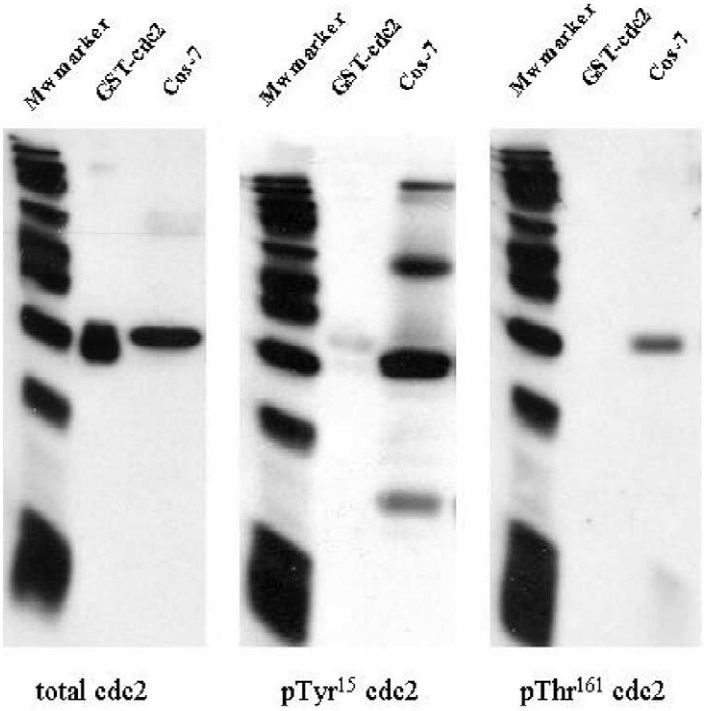

Fig. 2. Example of validation of phosphospecific antibodies. Total anti-cdc2 antibody recognizes both bacterially expressed (non-phosphorylated) GST-cdc2 fusion protein and cellular (COS-7, phosphorylated) cdc2, while phosphospecific antibodies detect only phosphorylated cdc2 protein from COS-7 cells. Anti-total cdc2 (clone POH-1), anti-pTyr^{15}cdc2 (≠9111) and anti-pThr^{161}cdc2 (≠9114) were from Cell Signaling Technology.

PKC) [36], splice variants (e.g., cdc25a and cdc25c) [37], well-conserved phosphoregions (e.g., Thr14/Tyr15 of cdk1, cdk2, and cdk5) [38], and SDS–PAGE mobility shifts induced by post-translational modifications (e.g., cdc25c) [39]. In addition to being a valuable tool for verifying phosphospecificity, 2D-E can be used to verify that the major immunoreactive protein by immunoblotting not only has the correct apparent molecular mass but also an appropriate p*I*. Moreover, mass spectrometry can be applied to conclusively identify an immunoblotting band [40], but this approach often requires the antibody to immunoprecipitate the protein in order to achieve workable concentrations. When knock-out cells/animals are available, using lysates from these can be useful in validating immunoreactive bands [41]. RNAi technology is quickly becoming a more readily available alternative to use of knock-outs for this purpose and will likely to further facilitate antibody characterization [42,43].

3.3. Study of cell cycle regulation using phosphorylation state specific antibodies

The cell division cycle is a complex process by which the cell divides into two viable daughter cells. In the progression from one phase of cell cycle to the next one, cells stop at several ''checkpoints'' in the cycle to query their internal state and external conditions. The progression from one phase to another is driven by phosphorylating enzymes, CDKs.

CDKs activators, the cyclins, however are unstable and are ''cycling'' during the cell cycle [44]. Thus, cyclins control the activities of CDKs and play a key role in cell cycle regulation. As cells proceed through the cycle, four major cyclins are produced sequentially (D, E, A, and B), and they activate CDKs. B-type cyclins associate with cdc2 to trigger mitosis. Progression through S phase requires cyclin A, presumably in association with cdk2. Cyclins D and E drive a cell into S-phase. The three D-type cyclins (cyclin D1, D2, D3) are very similar but they share very little homology with cyclin E. During cell cycle progression, D cyclins start accumulating at mid-G1, whereas cyclin E appears later, just prior to the G1/S transition.

After active cyclin D-dependent kinase (cdk4/6) is assembled in the nucleus, it phosphorylates RB protein [45], preventing its binding to E2F, thus inducing E2F-mediated transcription [46]. The E2F transcription factor activates genes whose products

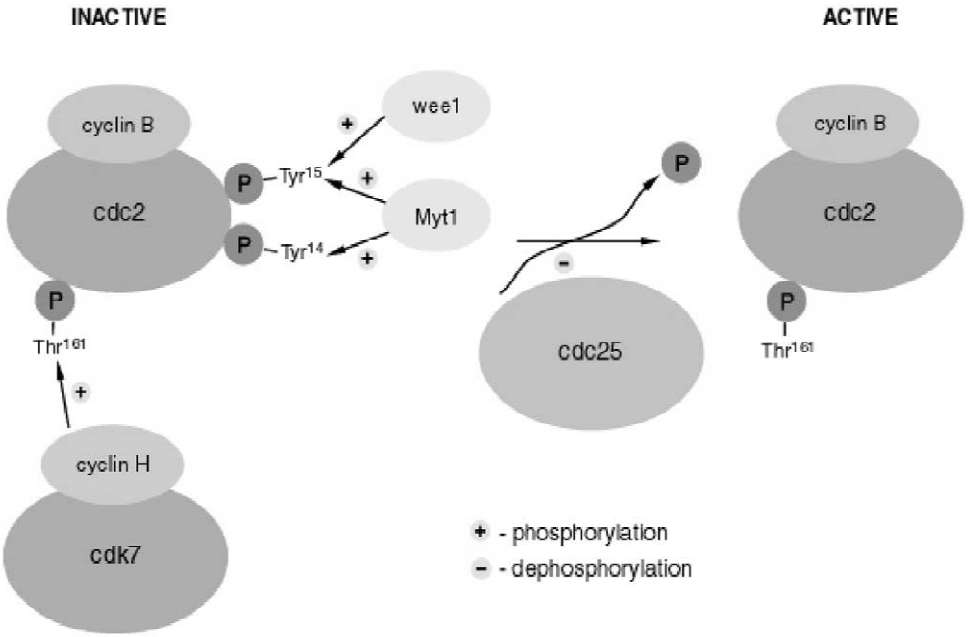

Fig. 3. Phosphorylation/dephosphorylation mechanisms controlling cdc2 activity.

are involved in nucleotide metabolism and DNA synthesis [47]. For understanding the restriction point, it is important to emphasize that E2F transactivates cyclins E and A [48]. Cyclin E enters into a complex with cdk2 and collaborates with cyclin D-cdk4/6 to complete RB phosphorylation. Cyclin E-cdk2 has a broader specificity than cyclin D-cdk4/6. For example, cyclin E-cdk2 phosphorylates the CDK inhibitor, p27, causing its degradation [49]. The activity of cyclin E-cdk2 is inhibited by p21, p27, and p57 [50]. In contrast, these CDK inhibitors, at least at low concentrations, co-activate cyclin D-CDKs. Another class of CDK inhibitors, p15, p16, and p18, specifically inhibit cyclin D-CDKs.

In addition to the regulation of CDK activity by association to its activating cyclin and/or inhibiting CDK inhibitor, at least two other mechanisms control CDK activity (Fig. 3):

(i) the activating phosphorylation of CDKs around position 161 is catalyzed by an enzyme called CAK (CDK-activating kinase). CAK is itself composed of the cdk7 complexed with cyclin H [51–54];

(ii) members of the cdc25 family of protein phosphatases which keep the complex in a inactive state due to the phosphorylation of Tyr^{14} and Tyr^{15} [53,55], which is catalyzed by the Wee1 and Myt1 kinases. For instance, cyclin B-cdc2 activation is triggered when cdc25 phosphatase dephosphorylates Tyr^{15}. In turn, the activity of cdc25 is regulated by both activating and inhibitory phosphorylations [53,54,56]. Phosphorylation of cdc25 at Ser^{216} by chk1 (a check point-activated kinase that participates in the G2-arrest of cells with damaged DNA) leads to the inactivation of cdc25, while phosphorylation by M-phase activated kinase, cdc2, creates a positive feedback loop leading to the rapid activation of the cyclin B-cdc2 complex. The combined effects of all these control mechanisms account for fine checkpoint regulation of cell cycle and cellular proliferative response to a variety of extracellular signals.

3.4. Multiple protein spots

Several regulatory phosphorylation sites on cdc2 result in multiple protein spots on 2-DE as evidenced by specific immunostaining (Fig. 4). Moreover, application of total versus phosphospecific cdc2 antibodies enabled us to distinguish unphosphorylated protein variant (the most basic spot) from Thr^{161} and/or Tyr^{15} phosphorylated variants.

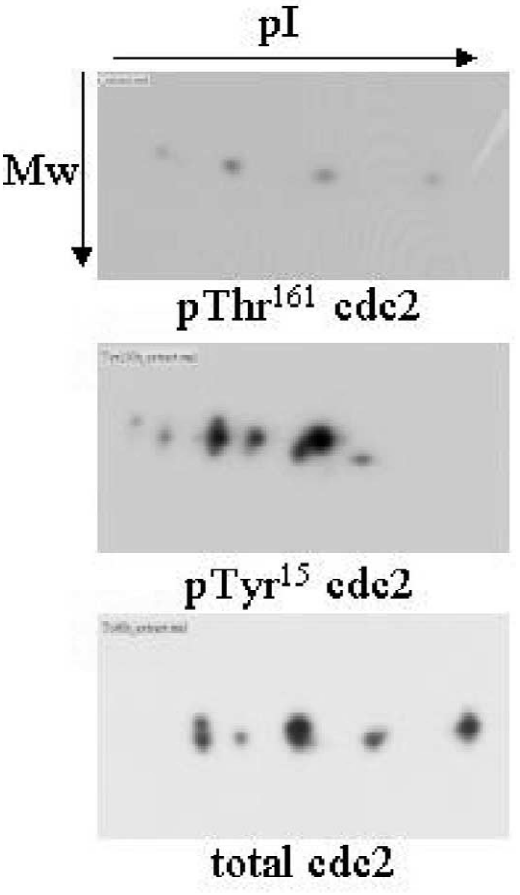

Fig. 4. Identification of multiple cdc2 variants in CEM T-lymphoblastic leukemia cells by 2-DE electrophoresis coupled to phosphorylation state-specific immunodetection. Anti-total cdc2 (clone POH-1), anti-pTyr^{15}cdc2 (≠9111) and anti-pThr^{161}cdc2 (≠9114) were from Cell Signaling Technology (horizontal arrow indicates direction of p*I* from acidic to basic part, vertical arrow shows decreasing molecular mass of 2-DE separation).

Due to pathological activation of CDKs in tumor cells, those proteins are considered as an ideal targets for new drugs. Series of synthetic CDK inhibitors were recently discovered and some of them are already in clinical trials [57,58]. These drugs are ATP analogs and selectively inhibit CDK activity by competitive binding to the ATP pocket of the kinase(s). Analysis of the effects of CDK inhibitors on malignant cells is an attractive area for application of various phosphoproteomic approaches.

In this paper, we demonstrate applicability of

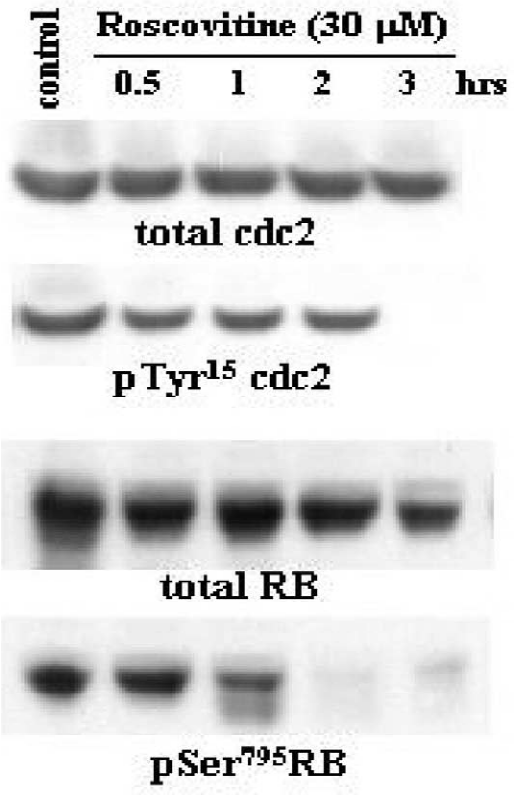

Fig. 5. Application of phosphoproteomics in identification of mechanisms of action of cell cycle therapeutics. Roscovitine (synthetic CDK inhibitor) inhibits phosphorylation of CDK substrate, RB protein, as evidenced by appearance of hypophosphorylated (low-molecular mass) RB on Western blots, and disappearance of Ser^{795} phosphorylated protein isoform in treated CEM cells. Moreover, the lack of cellular CDK activity induces activation of cdc2 via removing of inhibitory phosphorylation on Tyr^{15} residue. Anti-total cdc2 (clone POH-1), anti-pTyr^{15}cdc2 (≠9111), anti-RB (≠9309) and anti-Ser^{795}RB (≠9301) were from Cell Signaling Technology.

phosphospecific antibodies for study of RB and cdc2 phosphorylations (Fig. 5) in cells treated with synthetic CDK inhibitor roscovitine. Our results demonstrate that roscovitine-mediated CDK inhibition resulted in dephosphorylation of RB protein and the lack of cellular CDK activity was accompanied by compensatory dephosphorylation of inhibitory pTyr^{15} residue on cdc2 (Fig. 4).

4. Conclusion

Current findings demonstrate that 2-DE analysis coupled to immunoblot with protein phosphorylation state-specific antibodies represent a reasonable alternative to the use of other tools to study protein phosphorylation. The major advantage of the presented approach is its relative simplicity and sensitivity that allows specific detection of protein phosphorylation within complex protein mixture and distinguishes different phosphorylation sites of target protein. The method, once upgraded for a high throughput analysis with a panel of antibodies directed to specific regulatory or signaling pathways are expected to have clinical utility in the near future.

Acknowledgements

Support of this study by the Grant Agency of the Czech Republic (≠301/02/0475 and 303/02/0875), EU grant QLRT-1999-0014, MSMT grant J14/98 15110000, and Cancer Research Foundation in Olomouc is greatly acknowledged.

References

[1] H. Kovarova, R. Necasova, S. Porkertova, D. Radzioch, A. Macela, Proteomics 1 (2001) 587.
[2] T. Hunter, Cell 100 (2000) 113.
[3] K. Dua, T.M. Williams, L. Beretta, Proteomics 1 (2001) 1191.
[4] T. Hunter, Phil. Trans. R. Soc. B 353 (1998) 583.
[5] R. Patarca, Crit. Rev. Oncol. 7 (1996) 343.
[6] P. Cohen, Annu. Rev. Biochem. 58 (1989) 453.
[7] A. Fattaey, R.N. Booher, Prog. Cell. Cycle Res. 3 (1997) 233.
[8] B. Sebastian, A. Kakizuka, T. Hunter, Proc. Natl. Acad. Sci. USA 9 (1993) 3521.
[9] I. Humphery-Smith, S.J. Cordwell, W.P. Blackstock, Electrophoresis 18 (1997) 1217.
[10] T. Rabilloud, Proteomics 2 (2002) 3.
[11] H. Kovarova, P. Halada, P. Man, I. Golovliov, Z. Krocova, J. Spacek, S. Porkertova, R. Necasova, Proteomics 2 (2002) 85.
[12] M.P. Molloy, B.R. Herbert, B.J. Walsh, M.I. Tyler, M. Traini, J.-C. Sanchez, D.F. Hochstrasser, K.L. Williams, A.A. Gooley, Electrophoresis 19 (1998) 837.
[13] L. Tonella, C. Hoogland, P.-A. Binz, R.D. Appel, D.F. Hochstrasser, J.-C. Sanchez, Proteomics 1 (2001) 409.
[14] T.H. Steinberg, E. Chernokalskaya, K. Berggren, M.F. Lopez, Z. Diwu, R.P. Haugland, W.F. Patton, Electrophoresis 21 (2002) 486.
[15] D. Figeys, Proteomics 2 (2002) 373.
[16] T.J. Griffin, R. Aebersold, J. Biol. Chem. 276 (2001) 45497.

[17] A. Stensballe, S. Andersen, O.N. Jensen, Proteomics 1 (2001) 207.
[18] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotech. 20 (2002) 301.
[19] H. Zhou, J.D. Watts, R. Aebersold, Nat. Biotech. 19 (2001) 375.
[20] Y. Oda, T. Nagasu, B.T. Chait, Nat. Biotech. 19 (2001) 379.
[21] C.L. Chang, N. Hora, N. Huberman, R. Hinderer, M. KuKuruga, S.M. Hanash, Proteomics 1 (2001) 1415.
[22] M. Wind, M. Edler, N. Jakubowski, M. Linscheid, H. Wesch, W.D. Lehmann, Anal. Chem. 73 (2001) 29.
[23] S. Birkelund, L. Bini, V. Pallini, M. Sanchez-Campillo, S. Liberatori, J.D. Clausen, S. Ostergaard, A. Holm, G. Christiansen, Electrophoresis 18 (1997) 563.
[24] H. Is'harc, D. Watling, M. Kerr, Proteomics 1 (2001) 767.
[25] P.B. Maguire, K.J. Wynne, D.F. Harney, N.M. O'Donoghue, G. Stephens, D.J. Fitzgerald, Proteomics 2 (2000) 642.
[26] H. Kaufmann, J.E. Bailey, M. Fussenegger, Proteomics 1 (2001) 194.
[27] C.-J. Sherr, Cancer Res. 60 (2000) 3689.
[28] J. Bartek, J. Lukas, M. Strauss, Oncol. Rep. 3 (1996) 237.
[29] D.F. Stern, Exp. Mol. Pathol. 70 (2001) 327.
[30] H. Kovarova et al., Electrophoresis 21 (2000) 3757.
[31] T. Rabbiloud, Proteomics 2 (2002) 3.
[32] Q.P. Weng, M. Kozlowski, C. Belham, A. Zhang, M.J. Comb, J. Avruch, J. Biol. Chem. 273 (1998) 16621.
[33] H. Cha, P. Shapiro, J. Cell. Biol. 153 (2001) 1355.
[34] D.L. Foletti, R.H. Scheller, J. Neurosci. 21 (2001) 5461.
[35] A.C. Gingras, B. Raught, S.P. Gygi, A. Niedzwiecka, M. Miron, S.K. Burley, R.D. Polakiewicz, A. Wyslouch-Cieszynska, R. Aebersold, N. Sonenberg, Genes Dev. 5 (2001) 2852.
[36] H. Togashi, C.A. Hirshman, C.W. Emala, Am. J. Physiol. 272 (1997) L603.
[37] S. Wegener, W. Hampe, D. Herrmann, H.C. Schaller, Eur. J. Cell. Biol. 79 (2000) 810.
[38] L.R. Zukerberg, G.N. Patrick, M. Nikolic, S. Humbert, C.L. Wu, L.M. Lanier, F.B. Gertler, M. Vidal, R.A. Van Etten, L.H. Tsai, Neuron 26 (2000) 633.
[39] E.A. Kohn, N.D. Ruth, M.K. Brown, M. Livingstone, A. Eastman, J. Biol. Chem. 277 (2002) 26553.
[40] H. Zhang, Q. Wang, K. Kajino, M.I. Greene, DNA Cell. Biol. 19 (2000) 253.
[41] G. Schmid, Z.Q. Wang, J. Wesierska-Gadek, Biochem. Biophys. Res. Commun. 255 (1999) 399.
[42] S. Dutoya, S. Gibert, G. Lemercier, X. Santarelli, D. Baltz, T. Baltz, N.J. Bakalara, Biol. Chem. 276 (2001) 49117.
[43] P.A. Sharp, Genes Dev. 15 (2001) 485.
[44] D.M. Koepp, J.W. Harper, S.J. Elledge, Cell 97 (1999) 431.
[45] P.W. Hinds, S. Mittnacht, V. Dulic et al., Cell 70 (1992) 993.
[46] J.W. Harbour, D.C. Dean, Genes Dev. 14 (2000) 2393.
[47] J.R. Nevins, Cell Growth Diff. 9 (1998) 585.
[48] J. Bartek, J. Bartkova, J. Lukas, Curr. Opin. Cell Biol. 8 (1996) 805.
[49] R. Sheaff, M. Groudine, M. Gordon et al., Genes Dev. 11 (1997) 1464.
[50] C.J. Sherr, J. M Roberts, Genes Dev. 13 (1999) 1501.
[51] C. Norbury, P. Nurse, Annu. Rev. Biochem. 61 (1992) 441.
[52] S. Atherton-Fessler et al., Mol. Cell. Biol. 13 (1993) 1675.
[53] N. Watanabe et al., EMBO J. 14 (1995) 1878.
[54] K. Galaktionov et al., Genes. Dev. 9 (1995) 1046.
[55] T. Hunter, Cell 80 (1995) 225.
[56] C.H. McGowan, P. Russell, EMBO J. 12 (1993) 75.
[57] M. Hajduch, V. Noskova, G. Feketova, R. Novotny, K. Jess, L. Gojova, I. Kasparek, V. Krystof, J. Vesely, M. Strnad, V. Mihal, Drug resistance in leukemia and lymfoma III, in: R. Pieters, A. Veerman, G.J.L. Kaspers (Eds.), Kluwer Academic/Plenum Press, New York, 1999, p. 341.
[58] J.K. Buolamwini, Curr. Pharm. Des. 6 (2000) 379.