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A quantitative method for measuring protein phosphorylation[☆]

J. Andres Mckenzie and Phyllis R. Strauss*

Department of Biology, Northeastern University, Boston, MA 02115, USA

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Abstract

We have developed a novel method for quantitating protein phosphorylation by a variety of protein kinases. It can be used with purified kinases and their substrates *in vitro* or in combination with cell extracts. The method is based on the knowledge that protein kinase C (PKC) adds three phosphates to each molecule of its preferred substrate, myelin basic protein (MBP). A time course is performed in which a kinase is allowed to phosphorylate its preferred substrate or the protein under investigation in the presence of [γ -³²P]ATP. At the same time PKC is allowed to fully phosphorylate MBP. After resolving the products by SDS-PAGE, electrophoretic transfer, and determining the degree of incorporation of ³²P by phosphorImager analysis, the data are converted to moles phosphate/mole protein by normalization with phosphorylated MBP. The method is both sensitive and relatively rapid and all the steps are commonly available in the biochemistry laboratory. We have used this method to confirm and extend information on the relationship of MEK1 and MAPK/Erk2 in rat lung fibroblasts exposed to V₂O₅. A 4-h exposure to V₂O₅ results in partial phosphorylation of MAPK/Erk2 such that 25% of the potential phosphorylation sites are occupied. We also demonstrate that despite multiple potential phosphorylation sites, recombinant human AP endonuclease is weakly phosphorylated *in vitro* (4% at best) by PKC, cGMP-dependent protein kinase, casein kinase II, and casein kinase I and not at all phosphorylated by MAPK. Furthermore we are unable to demonstrate phosphorylation in cell extracts from HeLa cells, mouse fibroblasts after oxidative damage with H₂O₂ or alkylation damage with methylmethane sulfonate, or rat lung fibroblasts after oxidative damage with V₂O₅.

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Protein phosphorylation is the term employed to describe the reversible transfer of a phosphate group by a group of enzymes, collectively known as protein kinases, to target proteins in order to regulate their activity. Phosphate groups are usually added to serine, threonine, or tyrosine residues, although phosphorylation of histidine residues has been reported in some prokaryotic systems [1,2]. The removal of phosphate groups, or dephosphorylation, is carried out by protein phosphatases. Phosphorylation/dephosphorylation provides a fast, reversible, and efficient means of regulating complex pathways for such important biological activities as cell

cycle regulation, cell differentiation, DNA replication and repair, cell division and signaling, membrane transport, and metabolism [3].

Most protein kinases and phosphatases have multiple substrates *in vivo*, allowing them to influence and regulate multiple pathways. Furthermore, many kinases possess the ability to phosphorylate nonphysiological, *i.e.*, casein or peptide substrates, as well as physiological substrates. The relevance of phosphorylation of nonphysiological substrates remains unclear, as the ability to target a substrate *in vitro* does not necessarily indicate that such an interaction occurs *in vivo*. A good example involves some substrates for the casein kinases [4,5]. However, the degree to which a kinase phosphorylates its target protein, whether *in vivo* or *in vitro*, the number of phosphates that a kinase places on a single target molecule, and phosphate turnover have remained vexing problems. Without this information it is often difficult to quantitate individual steps in a kinase cascade. In one case, Ramwani et al. [6] and Ramwani and

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* Corresponding author. Fax: 1-617-373-2138.

E-mail address: p.strauss@neu.edu (P.R. Strauss).

Moscarello [7] demonstrated that protein kinase (PKC)¹ adds three phosphates to myelin basic protein (MBP). In another case, Wilsbacher et al. [8] demonstrated in vitro that MEK1 and MEK2 add 1.3 or 2.4 phosphates to Erk2, respectively. Each group sequenced the protein after it had been phosphorylated by [γ -³²P]ATP to quantitate the number of phosphates and identify the amino acid residues to which they added. However, the method is laborious and requires large amounts of both protein and kinase. In this study we present a method that may be used with ease and confidence to measure the degree of phosphorylation of individual proteins by purified kinases or cell extracts.

Materials and methods

Materials. Kinases were obtained from the commercial sources listed below. MBP was obtained from StressGen Biotechnologies (Victoria, BC), and histone H2B was purchased from Worthington Biochemicals (Lakewood, NJ). Human recombinant AP endo, expressed in *Escherichia coli* from the pXC53 expression vector, was purified as described [9]. Rabbit anti-human AP endo antibody was prepared at the Sealy Center for Molecular Science, University of Texas Medical Center, Galveston, Texas. Rabbit anti-mouse MAP kinase/Erk2 was obtained from Upstate Biotechnology (Lake Placid, NY). Protease and phosphatase inhibitor cocktails were purchased from Sigma–Aldrich (St. Louis, MO) or from Roche Diagnostics, GmbH (Mannheim, Germany). Phosphatase inhibitor cocktail 1, which inhibits L-isozymes of alkaline phosphatase as well as serine/threonine protein phosphatases such as PP1 and PP2A, contained microcystin LR, cantharidin, and (-)-*p*-bromotetraamisol in DMSO. Phosphatase inhibitor cocktail 2, which inhibits acid and alkaline phosphatase activities as well as tyrosine protein phosphatases, contained sodium orthovanadate, sodium molybdate, sodium tartarate, and imidazole in a pH 10.0 aqueous solution. HeLa cells were provided by Dr. Mark Perella (Harvard Medical School, Boston, MA); mouse fibroblasts were provided by Dr. Julie Horton (NIEHS, Research Triangle Park, NC), while primary passage rat lung fibroblasts were provided by Dr. James Bonner (NIEHS).

¹ *Abbreviations used:* AP endo, apurinic/aprimidinic endonuclease; CK I, casein kinase I; CK II, casein kinase II; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MAPK/Erk, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase/Erk kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NP-40, Nonidet P-40; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PS, phosphatidylserine; PVDF, polyvinyl difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

In vitro phosphorylation assays. In vitro phosphorylation assays were performed for 0–180 min in a 25- or 50- μ l volume under the conditions recommended by the commercial supplier of each kinase (see below). The specific activity of [γ -³²P]ATP was either 400 or 260 mCi/mmol. Each reaction was stopped by addition of EDTA (final concentration, 50 mM) and SDS (final concentration 0.1%) followed by vortexing. Eight microliters of 6 \times SDS protein loading buffer (0.35 M Tris–Cl, pH 6.8, 36% v/v glycerol, 10% w/v SDS, 0.6 M DTT, and 0.012% w/v bromphenol blue) was added for a final concentration of 1 \times loading buffer [9]. After boiling for 3 min, proteins were resolved by SDS–PAGE using a 10% SDS–PAGE gel electrophoresed at 200 V for approximately 2 h. The gel was stained with Coomassie blue, destained with 45% methanol/10% acetic acid, and dried on 3MM Whatman paper before being exposed to a phosphorImager screen for 2–15 h. The screen was then scanned with a Storm 840 phosphorImager (Molecular Dynamics, Sunnyvale, CA). Data analysis, quantitation, and graphing were performed using the programs Image Quant and KaleidaGraph, respectively. In early experiments we used the same amount of standard protein (MBP) as unknown substrate. In later experiments we observed that a difference of two orders of magnitude between MBP and the unknown still gave satisfactory results.

Phosphorylation with casein kinase II. Recombinant CK II (both α and β subunits derived from a human glioblastoma cDNA library) was obtained from New England Biolabs (Beverly, MA). Assay mix contained 0.1 mM ATP, 0.04 μ Ci/ μ l [γ -³²P]ATP, 20 mM Tris–HCl (pH 7.5), 50 mM KCl, and 10 mM MgCl₂. After 40 pmol of casein or 40 pmol of AP endo was added, the mixture was incubated at 37 °C with 10 units of CK II (1 unit is the amount of CK II required to catalyze the transfer of 1 pmol of phosphate to peptide substrate RRREEE–TEEE per minute at 30 °C in CK II buffer).

Phosphorylation with protein kinase C. PKC, purified from rat brain and containing a mixture of both α and β isozymes, was obtained from Promega (Madison, WI). Assay mix contained 0.15 mM ATP, 0.04 μ Ci/ μ l [γ -³²P]ATP, 50 mM Hepes–NaOH (pH 7.4), 10 mM MgCl₂, 2 mM CaCl₂, 1 mM DTT, and 0.6 mg/ml phosphatidylserine (PS). After the addition of 40 pmol of MBP or 40 pmol of AP endo, the reaction mix was incubated at 37 °C with 0.5 unit of PKC (1 unit is the amount of kinase needed to transfer 1 nmol of phosphate per minute using Type III-S histone as the substrate and phosphatidylserine at 0.6 mg/ml as activator).

Phosphorylation with cGMP-dependent protein kinase. The α isozyme of PKG-1, originally purified from rat liver, was obtained from Promega. Assay mix contained 0.2 mM ATP, 0.08 μ Ci/ μ l [γ -³²P]ATP, 20 mM MgOAc, 2 μ M cGMP, and 50 mM Hepes–NaOH (pH 7.4). After the addition of 40 pmol of histone H2B or 40 pmol of

AP endo, the mixture was incubated, for different periods of time at 30 °C with 100 unit of PKG (1 unit is defined as the amount of kinase required to incorporate 1 pmol of phosphate into the test heptapeptide RKRS-RAE per minute at 30 °C).

Phosphorylation with casein kinase I. CK I, obtained from Promega, was originally purified from rat liver tissue. Assay mix consisted of 0.1 mM ATP, 0.04 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP, 10 mM MgCl_2 , 40 μM EDTA, and 50 mM Hepes–NaOH (pH 7.4). After 40 pmol of casein or 40 pmol of AP endo was added, the mixture was incubated at 37 °C with 10 units of CK I (1 unit is defined as the amount of kinase needed to transfer 1 pmol of phosphate per min per mg at 37 °C using casein protein as the substrate).

Phosphorylation with MAPK. Recombinant MAPK(Erk2/p42) was obtained from New England BioLabs or Upstate Biotechnology. Assay mix contained 0.1 mM ATP, 0.04 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP, 50 mM Tris–HCl (pH 7.5), 10 mM MgCl_2 , 1 mM EGTA, 2 mM DTT, and 0.01% Brij 35. After 40 pmol of MBP or AP endo was added, the reaction mixture was incubated at 30 °C with 100 units of MAPK (1 unit is defined as the amount of MAP kinase required to catalyze the transfer of 1 pmol of phosphate to MBP in 1 min at 30 °C).

Phosphorylation with MEK1. Recombinant active MEK1 from Upstate Biotechnology used recombinant MAPK/Erk2 as substrate obtained from the same source. Assay mix contained 4 mM MOPS buffer, pH 7.5, 5 mM β -glycerol phosphate, 1.25 mM EGTA, 0.2 mM Na orthovanadate, 0.2 mM DTT, 10 mM MgCl_2 , 0.15 mM ATP, and 0.04 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP. The concentration of MAPK/Erk2 (0.5 ng/ μl) was chosen to approximate the concentration of MAPK/Erk2 that would be assayed in rat fibroblast cell lysates.

Phosphorylation assays using cell extracts. Growth of cell lines is described elsewhere [10,11]. In some cases HeLa cells were treated with phosphatase inhibitor cocktails for 1 h prior to cell lysis. Mouse fibroblasts were treated with no drug, 1 mM methylmethanesulfonate, or 15 μM H_2O_2 for 1 h and harvested 5 h later. Primary rat lung fibroblasts were grown to confluence, incubated with serum-free medium for 24 h and then exposed to medium with or without 400 μM V_2O_5 . These conditions are known to stimulate a variety of signaling pathways including MAP kinases, EGF receptors, transcription factors NF κ B, AP-1, and STAT-1 [10,12,13].

Cell extracts of mouse and rat fibroblasts, prepared by high salt lysis in the presence of protease and phosphatase inhibitor cocktails [14], were dialyzed to remove excess salt. Cell extract of HeLa cells was prepared by sonication in either triple-detergent lysis buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1 mM EDTA, 1% protease and phosphatase inhibitor cocktails 1 and/or 2, or no salt lysis

buffer (50 mM Hepes, 1% NP-40, 1% protease and phosphatase inhibitor cocktails 1 and/or 2). Lysates were stored at –80 °C until assayed. Endogenous kinase activity was examined by incubating approximately 50 μg of cell extract in the appropriate buffer containing [γ - ^{32}P]ATP and phosphatase inhibitor cocktail at 37 °C. Activity of exogenously added kinases was examined as described in other methods sections. Reactions were stopped by adding 6 \times protein loading buffer followed by boiling for 5 min before loading onto a 10% SDS–PAGE gel. Between 16 and 20 μg of total extract protein was loaded onto the gel. Alternately, reactions were stopped by adding EDTA (final concentration, 50 mM) plus SDS (final concentration, 0.1%) and stored at –20 °C until SDS–PAGE analysis.

Western blotting. Proteins were transferred onto polyvinylidene difluoride (PVDF) (Gelman Sciences, Dublin, Ireland) or nitrocellulose (Invitrogen Life Technologies, Rockville MD) membranes via electroblotting [15]. Rabbit anti-AP endo antibody (1:10,000 dilution) or rabbit anti-mouse MAPK/Erk2 (1:1000) was used as the primary antibody. The secondary antibody, goat anti-rabbit IgG HRP-conjugate diluted 1:3000 or goat anti-mouse IgG (H + L)-HRP conjugate (BioRad Laboratories, Hercules, CA) diluted 1:2000 was developed with Chemiluminescence reagent (NEN Life Science Products, Boston, MA) or with Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). The amount of AP endo or MAPK/Erk2 in the cell extract was calculated on the basis of a standard curve obtained by loading known amounts of the purified protein on the same SDS–PAGE gel with cell extract samples and analyzing the results as described above. Note that recombinant AP endo was quantitated during purification by the Bradford assay [16], which overestimates the amount of this protein 2.5-fold, as shown by end-group analysis [9]. Consequently, the protein concentration used here is the value determined by the Bradford assay and multiplied by 0.4. The concentration of MAPK/Erk2 was supplied by the manufacturer. To observe endogenous kinase activity, cell extract was incubated under the same conditions as the kinase of interest. In order to ensure that stretching or shrinkage of the gel did not introduce artifacts, proteins in the gel were first transferred to membranes. After the membranes were exposed to the phosphorImager screen to determine the migration of ^{32}P -phosphorylated proteins, they were treated with 1 0 and 2 0 antibodies to determine the precise location of MAPK/Erk2 or AP endo.

Results

Phosphorylation of preferred substrates can be used to quantitate moles phosphate incorporated into a substrate protein. In order to quantitate phosphorylation activity, we chose to examine the rate and degree of phosphor-

ylation by six different commercially available kinases. Casein was the known substrate for phosphorylation reactions with CK I and CK II; myelin basin protein served the same purpose for reactions with PKC and MAPK/Erk2; histone H2B was the known substrate for PKG; MAPK/Erk2 itself was used to assay MEK1. Assay conditions recommended by the supplier were employed in each case and the reaction was allowed to proceed for different periods of time up to 180 min in order to ensure that maximal phosphorylation was achieved. Time dependence of phosphorylation was readily observed (Figs. 1 and 2); the six kinases phosphorylated their preferred substrates in a time-dependent fashion and reached a plateau between 15 and 120 min after which time no further phosphorylation occurred. Figs. 1A and B (lanes 1–6) illustrate the time dependence of phosphorylation of PKC for its known substrate MBP and of CK II for its known substrate casein. PKC adds 3 mol of phosphate per mole of MBP [6,7]. Therefore, by using a known amount of MBP and test protein and a known specific activity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, one can calculate PIU per picomole of phosphate incorporated into MBP (PIU/pmol) and use this value to normalize the number of moles of phosphate incorporated into the unknown on a molar basis. Fig. 2 depicts the time dependence of phosphorylation by each kinase in the study, while Table 1 presents the data for all kinases at or near saturation. All of the substrates were phosphorylated with 0.25–2.3 mol phosphate per mole preferred substrate. MEK1 added 2.3 molecules phos-

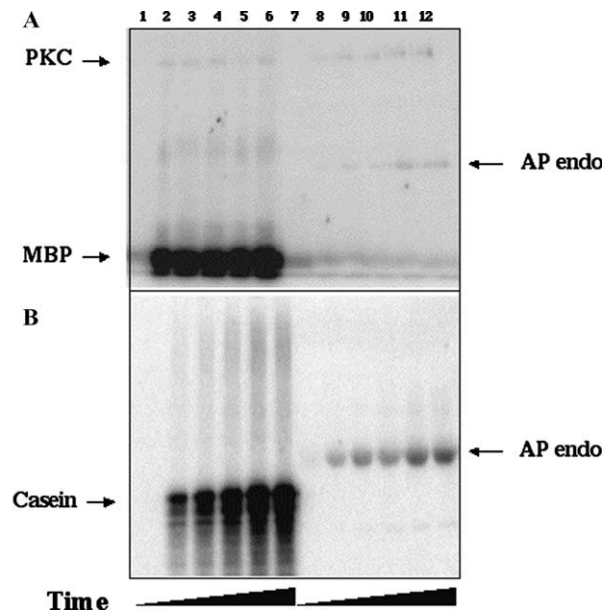


Fig. 1. Phosphorylation of preferred substrates and AP endo by PKC and CK II. (A) Time dependence of phosphorylation of MBP (lanes 1–6) and AP endo (lanes 7–12) by PKC. Forty picomoles of MBP (lanes 1–6) or AP endo (lanes 7–12) was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 0.5 units of PKC for 0, 15, 30, 60, 120, and 150 min, followed by SDS-PAGE electrophoresis. Lanes 1 and 7 contain MBP and AP endo, respectively, in the absence of kinase. (B) Time dependence of phosphorylation of casein (lanes 1–6) and AP endo (lanes 7–12) by CK II. Forty picomoles of casein (lanes 2–6) or AP endo (lanes 8–12) was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 10 units of CK II for 0, 15, 30, 60, 120, and 150 min, followed by SDS-PAGE electrophoresis. Lanes 1 and 7 contain casein and AP endo, respectively, in the absence of kinase.

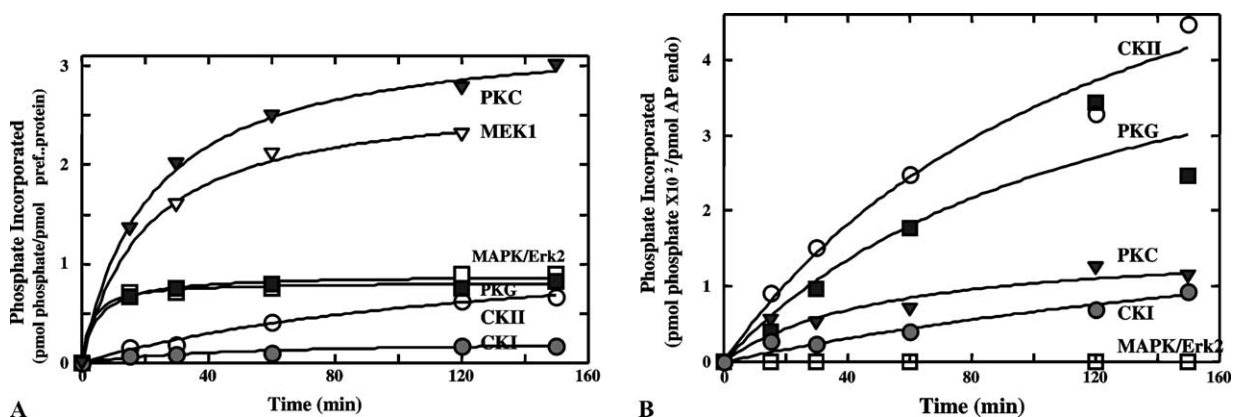


Fig. 2. (A) Quantitation of the phosphorylation of preferred substrates. Preferred substrate was mixed with the indicated kinase under conditions described under Materials and methods for the specified length of time. After resolution of protein by SDS-PAGE as shown in Fig. 1 and phosphorImager analysis, phosphorImager units (PIU) corresponding to phosphorylation of preferred substrate were divided by the molar amount of preferred substrate and converted to pmol phosphate/pmol substrate using the PKC standard included in each experiment. These curves represent the average of two or more experiments for each kinase. (B) Phosphorylation of AP endo by different kinases. AP endo (40 pmol) was mixed with the indicated kinase under conditions described under Materials and methods section for the specified length of time. After SDS-PAGE and phosphorImager analysis, phosphorImager units (PIU) corresponding to phosphorylation of preferred substrate were divided by the molar amount of preferred substrate and converted to pmol phosphate/pmol substrate using the PKC standard included in each experiment. These curves represent the average of two or more experiments for each kinase. The time dependence of phosphorylation of AP endo by each kinase was examined in the same experiment as time dependence of preferred protein substrate.

Table 1
Quantitation of degree of phosphorylation of preferred substrate and AP endo by five different protein kinases

Kinase	Preferred protein (moles phosphate/mole protein)	AP endo (moles phosphate/mole protein)
PKC	3.0	0.02
MEK1	2.3	ND
MAPK/Erk2	0.9	0.00
PKG	0.8	0.03
CK II	0.7	0.04
CK I	0.2	0.01

Purified kinases were incubated with [γ - 32 P]ATP and either preferred substrate or AP endo as described under Materials and methods. A control consisting of MBP incubated with PKC was included in each experiment as a means of calculating phosphorImager units per mole phosphate. The preferred protein for PKC and MAPK was MBP. The preferred protein for PKG was histone H2B, while casein was the preferred protein for CK I and CK II. The protein used to assay MEK1 was MAPK/Erk2. ND, not done.

phosphate per mole MAPK/Erk2. Note that MEK1 fully phosphorylated MAPK/Erk2 by 2 h. PKG and MAPK placed approximately one molecule phosphate onto each molecule of preferred substrate, CK II was somewhat less efficient, incorporating approximately one molecule phosphate/2 molecules casein, while CK I was the least efficient, placing one molecule of phosphate per 5 molecules of substrate. Thus, we have developed a method for quantitating the degree of phosphorylation of any protein, provided that a time course of phosphorylation is performed to ensure maximal modification of substrate and a standard protein with known degree of phosphorylation is included.

AP endo is weakly phosphorylated by CK II, PKC, PKG, and CK I and not at all by MAPK/Erk2. Phosphorylation alters the activity of a number of proteins involved in DNA repair [17–25]. Human AP endonuclease (AP endo), a critical enzyme in repair of oxidatively damaged DNA [26–29], has multiple potential phosphorylation sites for a variety of kinases. Since three groups including ourselves reported phosphorylation of AP endo [23–26] and partially conflicting data on the effects of phosphorylation on enzymatic activity [23–25], we chose to quantitate phosphorylation of this protein. Phosphorylation of purified AP endo by five of the six kinases described above was examined. CK II, PKC, PKG, and CK I all phosphorylated AP endo; however, the degree of phosphorylation was weak at best (Figs. 1A and B, lanes 7–12; Table 1). CK II achieved the greatest degree of phosphorylation at 4%, followed in descending order by PKG, PKC, and CK I. MAPK/Erk2 did not phosphorylate AP endo at all under these conditions. Time dependence and saturation were observed both for the known substrate in each case (shown above) and for AP endo. Because the substrate range for MEK is extremely narrow, we did not examine whether MEK1 phosphorylates AP endo. Note that

phosphorylated AP endo migrated with the same mobility as the bulk AP endo detected either by Coomassie blue or by Western blot analysis.

Phosphorylation by exogenously added kinases of proteins in cell extracts is readily quantifiable. To determine whether phosphorylation in cell extracts could be quantitated, we examined the ability of MEK1 to phosphorylate endogenous MAPK/Erk2 in cell extracts from primary rat lung fibroblasts. We also investigated whether prior treatment of the cells with 400 μ M V_2O_5 , which activates the MAP kinase cascade [10,13], would alter the results. Under these conditions, the cellular concentration of MAPK/Erk2 remains constant at 0.2 pmol/20 μ g cell extract protein, as determined by Western blot analysis using recombinant MAPK/Erk2 as standard (data not shown). Fig. 3 represents the phosphorImager image of incorporation of [32 P]phosphate into proteins from cells that were sham treated (lanes 1–4) or treated with 400 μ M V_2O_5 (lanes 5–9) for 4 h prior to harvest. The sample in lane 5, treated with EDTA + SDS prior to adding 32 P, demonstrates that there is no background incorporation. Lanes 3, 4, 8, and 9 contained exogenous MEK1, while lanes 1, 2, 5, 6, and 7 contained no exogenously added MEK 1. Thus, lanes 1, 2, 6, and 7 provide information on phosphorylation by endogenous kinases, which may or may not be related to the ones under study here. In order to obtain the results presented in Fig. 4, PIU in the MAPK/Erk2 band

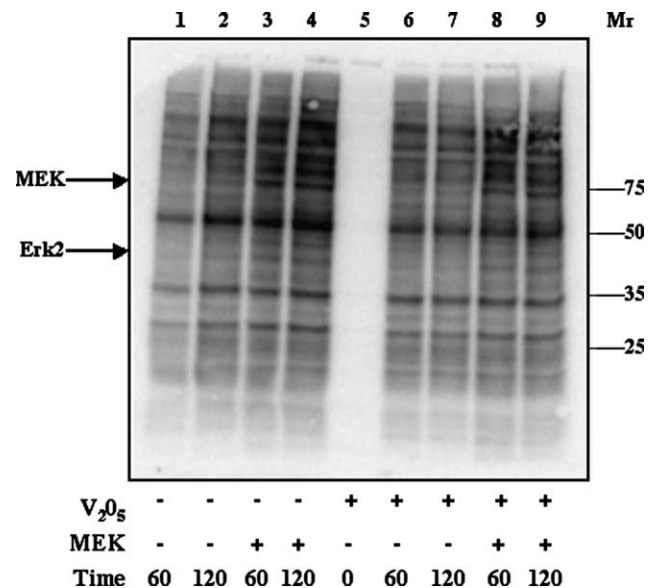


Fig. 3. Phosphorylation by exogenous MEK1 of MAPK/Erk2 in cell extracts. Stationary phase primary rat lung fibroblasts were treated with buffer (sham) or 400 μ M V_2O_5 for 4 h before lysis. After preparation of extracts, the ability of 0.5 U activated MEK1 to phosphorylate endogenous MAPK/Erk2 was examined. Lanes 1–4, extract from sham-treated cells; lanes 5–9, extract from V_2O_5 treated cells. MEK1 was added to cell extracts resolved in lanes 3, 4, 8, and 9. Extracts in lane 5 were treated with EDTA and SDS prior to addition of [γ - 32 P]ATP. A similar control for unstimulated cells is not shown.

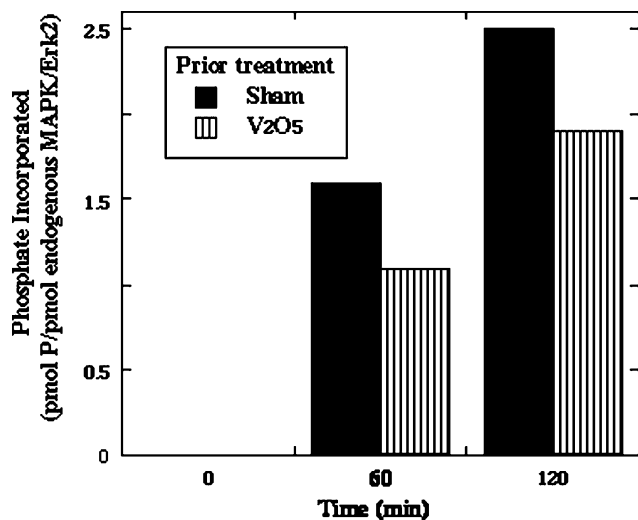


Fig. 4. Quantitation of phosphorylation of endogenous MAPK/Erk2 by exogenously added activated MEK1. The data are the average of two experiments.

from cell extracts from sham-treated cells was subtracted from PIU obtained from the MAPK/Erk2 band of cell extracts from cells treated with V_2O_5 . Since endogenous MEK1 has been activated prior to cell lysis in cells treated with V_2O_5 , we expect that some of the endogenous MAPK/Erk2 is already phosphorylated. Further, as the cell extract is prepared in a battery of phosphatase inhibitors, we expect that the phosphate will remain bound and that those sites will be unavailable to exogenous MEK1. Hence, the level of MAPK/Erk2 phosphorylation occurring during incubation with [^{32}P]ATP will be *less* with cell extract from V_2O_5 -treated cells than that in extract from sham-treated cells. The decrease allows one to estimate ongoing phosphorylation; that is, what percentage of phosphorylation sites are already occupied in MAPK/Erk2 in V_2O_5 cells. MEK1 clearly phosphorylates MAPK/Erk2 from both treated and untreated cell extracts, as shown in Fig. 3. As predicted, MEK1 adds 2.6 phosphates/mol endogenous MAPK/Erk2 in cell extracts from sham-treated cells in a time-dependent fashion, while the kinase adds 1.9 phosphates/mol MAPK/Erk2 in cell lysates from cells exposed to V_2O_5 during the same time interval. Since the level of phosphorylation of MAPK/Erk2 by exogenous MEK1 in extracts from V_2O_5 -treated cells is 75% that in extracts from sham-treated cells, 75% of phosphorylation sites are still available to MEK1. Therefore, a 4-h exposure to V_2O_5 results in phosphorylation of 25% available MAPK/Erk2 sites.

The 71-kDa band which is phosphorylated in the presence of MEK1 is likely to be the GST-fusion MEK1 itself, which is capable of autophosphorylation [30]. Quantitation of phosphates incorporated per mole MEK1 indicates that approximately 1 phosphate is added after 60 min incubation and close to 2 phosphates

are added after an additional 60 min. MEK1 also phosphorylates MAPK/Erk1. Although we did not quantitate the amount of MAPK/Erk1 in primary rat lung fibroblasts for this study, the phosphorylation pattern of MAPK/Erk1 followed the same pattern as phosphorylation of MAPK/Erk2.

Cell extracts from cells subjected to oxidative or methylation conditions are unable to phosphorylate or enhance phosphorylation of AP endo. Having demonstrated that recombinant AP endo is phosphorylated in vitro weakly at best, we turned our attention to examining phosphorylation of AP endo in a variety of cell extracts. Multiple attempts to identify phosphorylation of endogenous AP endo in cell extracts from mouse fibroblasts treated with $15\mu M H_2O_2$ or 1 mM methylmethanesulfonate were unsuccessful. The conditions were chosen because they decrease cell growth by 50% when cells are harvested after 24 h [11]. ^{32}P -AP endo, previously prepared as the standard by phosphorylation with CK II, appeared as a band with MW_{app} of approximately 34 kDa. At least 13 phosphorylated proteins appeared as specific bands in the cell extract after incubation under CK II or MEK1 conditions with [γ - ^{32}P]ATP and phosphatase inhibitors (data not shown). None of these corresponded to AP endo by Western blot analysis and none corresponded to the ^{32}P -phosphorylated AP endo prepared by phosphorylation with CK II. Concentration by immunoprecipitation of endogenous or exogenous AP endo previously exposed to kinase conditions with anti-AP endo antibodies and protein A-Sepharose beads followed by Western blot analysis also failed to detect phosphorylation. Furthermore, addition of HeLa cell extract to recombinant AP endo failed to stimulate phosphorylation by CK II (data not shown).

Discussion

The preceding data demonstrate that the degree of phosphorylation of a known or an unknown protein by a variety of kinases can be quantitated on a molar basis by including a phosphorylated standard with each experiment. The standard employed here uses PKC to phosphorylate myelin basic protein. Since the number of phosphates placed on each MBP by PKC is known, it is a simple matter to convert the phosphorImager data to moles phosphate, provided that the standard substrate and the unknown are present at known protein concentrations. An initial time course of PKC acting on MBP is required to determine the time interval to achieve maximal phosphorylation. Furthermore, in cases where phosphorylation is weak it is desirable to include time courses of phosphorylation of a standard protein as a positive control for kinase activity. Since there is no need to excise bands from polyacrylamide gels for quantitation by scintillation spectrometry, the method is relatively ef-

ficient, and it can be performed with equipment in the standard biochemistry laboratory.

The method we present here is more reliable than conventional protocols for two reasons. First, it employs a standard protein and kinase where a known number of residues on a molar basis are phosphorylated on the standard protein. Second, the time course ensures that maximal phosphorylation has occurred for both the standard and the unknown protein with the kinase in question. Therefore, it is possible to quantify even low levels of phosphorylation on a novel substrate.

Quantitation of phosphorylation of proteins in cell extracts can also be performed. However, if the protein is already phosphorylated in the cell and phosphatase inhibitors are present during preparation, the protein will fail to be acted upon by exogenous or endogenous kinases. On the other hand, these data demonstrate that it is a simple matter to measure incorporation of additional phosphates to partially phosphorylated endogenous proteins.

APendo, a key enzyme in the DNA base excision repair pathway [28,29,31], has a variety of potential phosphorylation sites that make it a candidate for regulation by phosphorylation. Yacoub et al. [23], Hsieh et al. [24], Fritz and Kaina [25] and we ourselves [26] have reported in vitro phosphorylation of APendo. The casein kinases and PKC employed were the same type and source as those used here. The ability of APendo to promote binding of transcription factors Fos and Jun is enhanced [32,33], while there are conflicting reports of inhibition of endonuclease activity. Our quantitative studies presented here indicate that in vitro recombinant APendo was a poor substrate for phosphorylation. We also could not detect phosphorylation in cell extracts of several cell lines exposed to oxidative stress conditions. We note that under these conditions we would not have been able to detect prior phosphorylation especially if the percentage were small.

In conclusion, we have developed a novel method for quantitating protein phosphorylation. The method is sensitive and rapid and the instrumentation is readily available.

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