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PROSPECTS

Approach to Systematic Analysis of Serine/Threonine Phosphoproteome Using *Beta* Elimination and Subsequent Side Effects: Intramolecular Linkage and/or Racemisation

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Abstract Complete analysis of the phosphorylation of serine and threonine residues directly from biological extracts is still at an early stage and will remain a challenging goal for many years. Analysis of phosphorylated proteins and identification of the phosphorylated sites in a crude biological extract is a major topic in proteomics, since phosphorylation plays a dominant role in post-translational protein modification. *Beta* elimination of the serine/threonine-bound phosphate by alkali action generates (methyl)dehydroalanine. The reactivity of this group susceptible of nucleophilic attacks might be used as a tool for phosphoproteome analysis. Most of the known serine/threonine kinases recognize motifs in protein targets that are rich in lysine(s) and/or arginine(s). The (methyl)dehydroalanine resulting from *beta* elimination of the serine/threonine-bound phosphate by alkali action is likely to react with the amino groups of these neighboring amino acids. Furthermore, the addition reaction of dehydroalanine-peptides with a nucleophilic group more likely generates diastereoisomers derivatives. The internal cyclic bonds and/or the stereoisomer peptide derivatives thus generated confer resistance to trypsin cleavage and/or constitute stop signals for exopeptidases such as carboxypeptidase. This might form the basis of a method to facilitate the systematic identification of phosphorylated peptides. J. Cell. Biochem. 100: 875–882, 2007. © 2006 Wiley-Liss, Inc.

Key words: kinase; serine/threonine phosphate; phospho-proteome

ARTICLE TEXT

The cellular homeostasis of the phosphoproteome, involving a complex interplay of kinases and phosphatases, has been extremely difficult to analyze. In the past, the permeability of membranes to inorganic ^{32}P was extensively exploited; phospho-proteins were radiolabeled within the cell through the production of radioactive ATP. This approach has been abandoned because of safety issues and contamination of analytical instruments. More recently, a two-step approach has been used instead: (i) isolation of phosphopeptides from a large collection of proteolytic fragments by immobilized metal affinity chromatography; (ii) identification of the phosphopeptides (by the 80 Da shift after dephosphorylation with alkaline phosphatase) using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [Larsen et al., 2001; Chen et al., 2002; Bonnefant et al., 2003]. However, the phosphate group tends to dissociate from the serine/threonine residues during MS (or tandem MS) analysis, which frustrates efforts to identify the phosphorylation site [Conrads et al., 2002]. No single approach has proved capable of identifying the complete panel of phosphopeptides in a tryptic digest of a complex mixture and/or extract. More notably, the basic residues near the phosphorylated residue hamper efficient alkaline phosphatase hydrolysis, which makes the 80 Da shift difficult to analyze by mass

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spectrometry. Moreover, the use of metal affinity columns to isolate phosphopeptides results in contamination with a large spectrum of non-phosphorylated species although this problem has been mitigated by converting the interfering carboxylic groups in methyl esters [Ficarro et al., 2002]. This shows how difficult it is to construct an extensive map of the phosphorylated sites in a crude cellular extract, and raises concerns about the attainability of this goal using existing proteome technology.

METHOD FOR SERINE/THREONINE PHOSPHOPROTEOME ANALYSIS

However, as it is essential to isolate the phosphopeptides first in order to identify them, new methods have been developed to enrich phosphopeptide preparations. These methods involve the substitution of a tag for the phosphate. The isolated tagged peptides are then analyzed using reversed phase liquid chromatography (LC) coupled directly on-line to MS (or tandem MS). Substitution of a tag for the phosphate moiety of a phospho-serine and/or threonine entails two steps:

(i) *beta* elimination of the phosphate by hydroxide ions, resulting in conversion of the residue to dehydroalanine or β methyl dehydroalanine. The newly-formed α β unsaturated double bond makes the β carbon sensitive to nucleophilic attack (see Table IA);

(ii) a Michael-type addition reaction with a nucleophile (amino group or thiol), which leads to covalent modification [Goshe et al., 2001; Oda et al., 2001; Conrads et al., 2002; Knight et al., 2003].

In this report we suggest that nucleophilic attack is most likely to come from the amino groups near the phosphorylated sites, because most serine/threonine kinases recognize highly basic consensus sequences. (Table IB indicates representative substrates for major serine/threonine kinases and the sequence motifs that determine the specificity). We suggest that the *beta* elimination described above can create an intramolecular linkage, which constitutes a resistance point for trypsin and/or carboxypeptidase hydrolysis. This could be used as a tool for separating phosphorylated peptides from their unphosphorylated forms.

PROPOSAL FOR A TECHNIQUE AND PRELIMINARY ASSESSMENT

We tried first to derivatize the phosphate groups directly on membranes after gel electrophoresis followed by Western blotting. The aim was to eliminate any artifacts of phosphorylation and/or phosphatase action during the extraction process. We found that the phosphate moieties of proteins blotted onto PVDF could be substituted in situ with biotin ethylene diamine. The serine and/or threonine phosphate was removed by alkali treatment of the membrane, and the unstable double carbon bond generated was submitted to nucleophilic attack by the biotin ethylene diamine amino group(s). *Beta* elimination of phosphate is faster than the subsequent covalent attachment, and faster than cysteinyl residue modification and alkaline hydrolysis of the amide bonds [Zhou et al., 2001]. The Michael addition reaction on dehydroalanine, although effective in alkali, also occurs at neutral pH, so we tried to *beta*-eliminate the phosphate first, then to attach covalently the biotinyl arm directly on membrane, carrying out the Michael addition at pH 8. We therefore treated the membranes with 1 M NaOH for 30 min to generate (methyl) dehydroalanine, then briefly washed it with water and incubated it for 24 h with 1 mM biotin/biotin ethylene diamine (10:1) in 20 mM phosphate buffer, pH 8.0 (see Fig. 1A).

The biotinyl arm allows bands to be revealed calorimetrically using avidin-alkaline phosphatase (see Fig. 1A). The *Drosophila* strains *rut* (cyclase mutated) and *dnc* (phosphodiesterase mutated) respectively presenting low and high levels of cAMP [Quinn et al., 1974; Tully and Quinn, 1985] and high level (*RQED*) and low level (*ala*) of Cam kinase II activity [Broughton et al., 1996], were tested using this protocol. The aim was to evaluate the differences in kinase activities in living flies (Fig. 1A). We observed unchanged patterns, which were expected in view of the sophisticated mechanisms of genomic regulation and the functional redundancy of many kinases.

We also found that proteins directly substituted with biotin in situ can be cleaved with trypsin (see Fig. 1B) and the peptides released can be collected directly from PVDF membranes. This enabled us to cut out bands of membrane corresponding to the molecular weights of interest. The peptides released by

TABLE I. β Elimination of Phosphate and Motif Sequence for Ser/Threo Phosphorylation Sites

A

$$—CHX—O—P(=O)(O^-)—O^- \longrightarrow PO_4^{3-} + \;\diagdown CH{=}CHX$$

Phospho-serine/threonine → **(methyl)dehydroalanine**

X: H (serine) or $\mathbf{CH_3}$ (threonine

B

Sequence	Protein
PKA	
KAKTRSS*RA	Histone H2A
GKKRKRS*RK	Histone H2B
ERRKS*KSGAG	ARPP-21
AVRRS*DRA	Troponin I
QWPRRAS*CTS	Glycogen synthase 1a
KRKRKS*S*QCL	c-erbA, v-erbA
YLRRAS*LAQLT	Pyruvate kinase
RTKRSGS*VYE	Phosphorylase kinase (*beta* chain)
AGARRKAS*GP	Histone H1
YLRRRLS*DSN	Synapsin I
NYRGYS*LGNY	Lysozyme
RAS*FGSRGS*GS	Desmin
PLSRTLS*VSS	Glycogen synthase
IRRRRPT*PAT	Phosphatase inhibitor
KPRRKDT*PAL	G protein
RFDRRVS*VCA	Subunit R2 of PKA
PKG	
DGKKRKRS*RK	Histone H2B
FRRLS*ISTE	Phosphorylase kinase
RRRRGAIS*AEVY	Subunit R1 PKA
KKPRRKDT*PAL	G protein
RRRRPT*PAML	DARPP-32
QIRRRRPT*PAT	Phosphatase inhibitor
PKRKVS*AEG	
KRRSARLS*AK	
Cam Kinase I	
NYLRRRLS*DS	Synapsin 1
Cam kinase II	
PSRTLS*VSS	Glycogen synthase
KMARVFS*VL	Calcineurin
YLRRAS*VAQL	Pyruvate kinase
RSKYLAS*AST	Myelin
PKC	
KKNGRVLT*LPRS	Insulin receptor
S*S*KRAKAKT*TKKR	Myosin
KKKKKRFS*FKKS*FKLSGFS*FKKNKK	MARCKS protein
RRRHIVRKRT*LRRL	EGF receptor
RRRHIVRKRT*LRRL	erb B
GSSKSKPKDPS*QRRRS	$pp60^{src}$
Myosin light chain kinase	
KRRAAEGSS*NVF	Myosin light chain
Myosin heavy chain kinase	
KAGT*TYALNLNK	Myosin heavy chain
CDC28 protein kinase	
HS*TPPKKKRKSSS*P	Large T antigen
KT*PK	Histone H1
Casein kinase II	
ADS*ES*EDEED	Subunit R2 PKA
VEEDAES*EDEDEED	Nuclear protein B23
EIEDVGS*DEE	Hsp (*alpha*)

R/K: sequence rich in amino groups, which are candidates for nucleophilic attack on the (methyl) dehydroalanine generated by alkali treatment from phosphoserine and/or phosphothreonine at the phosphorylation site.

AG: hydrophobic sequence or residue(s) generated by *beta* elimination of the phosphate and formation of (methyl) dehydroalanine.

C cysteine, presenting an SH group likely to effect a nucleophilic attack on (methyl) dehydroalanine.

D/E: sequence rich in carboxylic groups, weakly nucleophilic and therefore weakly reactive with (methyl)dehydroalanine.

These sequences are drawn from tabulations published in *Methods in Enzymology* by Pearson and Kemp [1991].

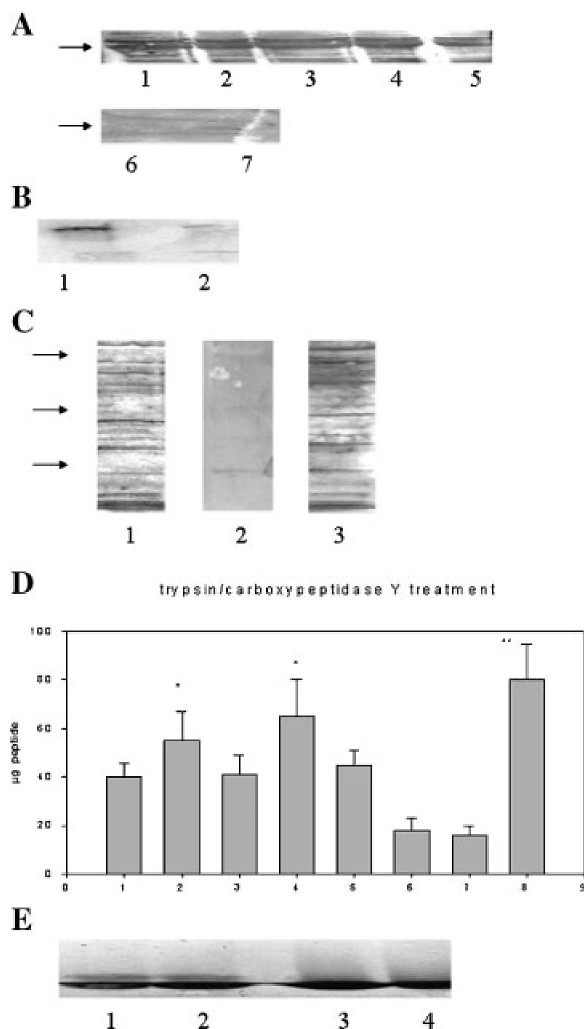


Fig. 1. Labeling of phosphoproteins on nitrocellulose and PVDF blot and quantification of intramolecular linkage after *beta* elimination of phosphate *Drosophila* heads were ground in SDS 1%. An aliquot was submitted to 7.5% acrylamide gel electrophoresis and the proteins were transferred by Western blotting to a nitrocellulose or PVDF membrane. Membranes were treated for derivatization of phosphoproteins as follows: PVDF or nitrocellulose membranes were treated with alkali (1 M NaOH for 30 min) then washed in 20 mM phosphate buffer, pH 7.4 and incubated with 10 mM biotin ethylene diamine (10:1) 10 mM for 24 h. Bands were revealed with BCIP/NBT alkaline phosphatase substrate (Sigma). **A**: PVDF membrane after labeling show two bands of references: ala (1) RQED (2) rut (3) dnc (4) wild type (5) control without NaOH treatment (6) control without biotin ethylene diamine incubation(7). Arrow indicates 50 kDa band. **B**: biotinylated peptides released by the action of trypsin on PVDF-blotted 50 kDa proteins previously submitted to biotin derivatization, were incubated with fluorescein-avidin for 30 min in 20 mM phosphate, pH 8.0, and submitted to 7.5% acrylamide gel electrophoresis followed by Western blotting on to nitrocellulose. The nitrocellulose membrane was then treated with methanol to make it transparent. A fluorescence-based photograph was obtained with a fluorescence imaging apparatus (1). A control without NaOH treatment of membrane is shown (2). **C**: Same experiment as in A except a full gel is shown with the extract of 10 heads each. Nitrocellulose membrane (1) nitrocellulose membrane (after biotin incubation) was treated with 10 µg/ml trypsin in 10 ml for 1 h at room temperature (2) PVDF membrane (3). Arrows indicate 205, 66 and 29 kDa bands. **D**: quantification of the peptides generated from crude head extract submitted to A elimination protocol and subsequent auto alkylation to generate putative intra-molecular linkage. The void volume of Biorad chromatography columns (Sephadex G10 exclusion size <1,000) was analyzed with the Biorad reagent for colorimetric peptide quantification (individual amino acids and di and tripeptides do not interfere with Coomassie Blue). ala2 (1), RQED (2), rut (3), dnc (4), NaOH/cysteamine (5), control without any treatment (6) Trypsin/carboxypeptidase control (7), phorbol/brcAMP/phosphatase inhibitors (8). * and **correspond respectively to $P < 0.08$ and 0.005 compared to the precedent column. **E**: Extract of fly head was treated with 1 M NaOH for 1 h and then neutralized with HCL. Salts were removed by membrane filtration and extract was kept for 24 h in 20 mM phosphate, pH 8.0. The samples were then digested with trypsin (50 µg/ml 1 h pH 7) and carboxypeptidase Y (75 µg/ml, 1 h pH 5.5) at 37°C and an aliquot was loaded on a 16% acrylamide gel. Independent duplicate of the experiment (1,2), independent duplicate of the control without alkali treatment (3,4).

trypsin from the blotted proteins can be concentrated with avidin agarose for further analysis. Moreover, we carried out the following experiment: the biotinyl-peptides released by trypsin from a blotted 50 kDa band were incubated with tetrameric fluorescein/avidin and analyzed by acrylamide gel electrophoresis. Tetrameric avidin has an affinity for biotin above 10^{-13} M, with four biotin binding sites resistant to SDS. The gel was blotted on nitrocellulose for fluorescence imaging. Dried nitrocellulose membranes become transparent upon treatment with ethyl acetate and/or methanol, keeping the blotted molecules unchanged (Fig. 1B). This allowed highly efficient fluorescence imaging detection and analysis when the membranes were incubated with a fluorescent probe such as antibody and/or avidin.

Proteins were also blotted on nitrocellulose membranes and were derivatized using the same protocol. As known, high concentrations of alkali (NaOH $>$ 1 M) destroy the nitrocellulose membrane after few hours, whereas the PVDF membrane is highly resistant but proteins are washed out. Furthermore, prolonged incubation with alkali hydrolyzed proteins on both types of membrane (PVDF or nitrocellulose) and drastically diminished the efficiency of derivatization. Bands appeared on the nitrocellulose membrane even when alkali treatment was short and mild (30 min and 0.1 N NaOH) but not all the candidates were revealed: the use of this membrane only reveals the major phosphoproteins.

This argues for a strategy of sequential steps, the first carried out in a high concentration of alkali for a short time, the second the derivatization step at pH 8.0 and at low salt concentration. Interestingly, this overall procedure allows the heads of *Drosophila* (or any living material) to be ground in SDS, thus preserving the phosphorylation state of the proteins and eliminating unwelcome phosphatase and/or kinase actions, which can occur because of contact among molecules from different cellular compartments during the extraction procedure.

INTRA MOLECULAR LINKAGE MIGHT BE USED TO ISOLATE RESISTANT PEPTIDES TO ENZYMATIC DIGESTION

We also treated both membrane-bound and soluble proteins with alkali/SDS solution in a test tube, as described in literature. In addition, fly heads were ground in 1 M NaOH for 1 h without SDS in the presence of a cocktail of phosphatase inhibitors. Biotin ethylene diamine was added simultaneously or sequentially; in the latter case, salts were eliminated first by filtration on *Millipore* membranes. Because the *beta* elimination is faster than covalent attachment by the Michael reaction, we obtained better results when the two steps were performed sequentially. However, even when this protocol was used, Western blots unexpectedly showed poorly biotinylated bands, leading us to conclude that the procedure was inefficient. Moreover, the proteins were extensively hydrolyzed after few hours in 1 M NaOH, generating small sized peptides. In order to understand the discrepancy in labeling between the two approaches (derivatization of crude extract and derivatization of PVDF membrane blotted proteins), we postulated that the membrane-blotted proteins are rigid, not free to rotate about the α-carbon bonds of the amide groups. In consequence, attack by exogenous soluble nucleophilic groups on the (methyl) dehydroalanine sites was favored over attack by endogenous nucleophilic residues. In support of this hypothesis, nucleophilic attacks are known to be more efficient in hydrophobic environment. Hydrogen bonds between nucleophilic groups and water molecules (water solvation) are believed to slow the reaction. Unprotonated amino groups are also more efficient than protonated ones. The serine and/or threonine targets of kinases are surrounded by residues rich in amino groups (see Table IB). The dehydroalanine remaining after *beta* elimination of the phosphate is hydrophobic. We observed that most of the serine and/or threonine targets of kinases are also flanked by hydrophobic residue(s) (see Table IB). We hypothesized that the few hydrophobic residues around the dehydroalanine create a pocket close to the lysine and/or arginine amino groups. This hydrophobic pocket might act as a pseudo-active site, where the aliphatic chain of lysine and/or arginine could position the amino group in front of the (methyl) dehydroalanine, resulting in a nucleophilic attack. These residues might compete favorably against exogenous nucleophilic molecules. We observed that amino groups of the other proteins interfere weakly with dehydroalanine. The PKA phosphorylated *Drosophila* head extract submitted to *beta*

TABLE II. (A) *Beta* Elimination of ^{32}Phosphate From Proteins of *Drosophila* Head Extract Phosphorylated by PKA*, (B) Covalent Binding of ^{32}Phosphate Histone H2B to (methyl) Dehydroalanine-Proteins of *Drosophila* Head Extract Generated by Alkali Treatment**

(A) Time (min)	cpm	
0	10,000	
10	8500 ± 350	
30	6500 ± 450	
60	1450 ± 350	
(B) Time (min) (alkali treatment)	^{32}P histone H2B/protein complex	putative total dehydroalanine component
0	1.5 ± 0.5 nmole	85 ± 12.5 nmole
10	2.5 ± 2.5 nmole	—
30	4.5 ± 2.5 nmole	—
60	12.5 ± 5.5 nmole	—

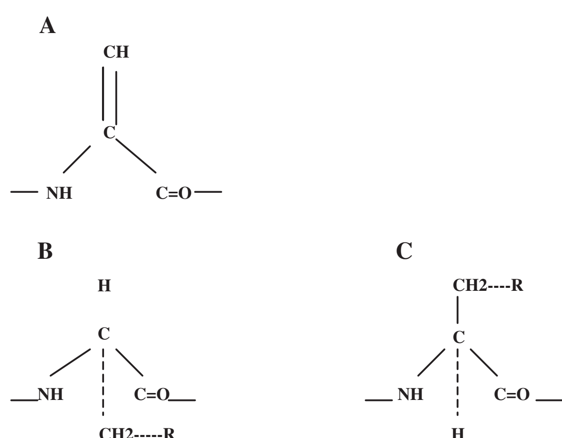
*Five milligrams soluble proteins were phosphorylated with PKA (100 units Sigma P 2645) and ^{32}Pγ-ATP 10 μM (10,000 cpm/nmol) in presence of cocktail of inhibitors of phosphatase (Sigma P 2850) 10 μl/ml for 30 min at 25°C. The extract was then submitted to alkali treatment (1 M NaOH) for the indicated time then neutralized and concentrated on *Millipore* membrane as described above. The sample was filtered on Sephadex G 10 column and an aliquot of the void volume was counted (the volume of the aliquot was arbitrary determined to give 10,000 cpm at $t = 0$ for each determination). Numbers are the mean ± SEM of three experiments. As expected we observed a decrease of radioactivity bound to proteins proportional to the duration of alkali treatment.
**After alkali treatment, proteins were desalted and incubated with ^{32}P histone H2B at pH = 8 for 24 h. The quantities of the covalent complex(s) were deduced after acrylamide electrophoresis separation and counting of specific activity. Numbers are the mean ± SEM of three experiments.

elimination showed poor efficiency to bind covalently added histone. Only 20% of putative sites were covalently bound to histone (see Table II). Our data suggest strongly that the intramolecular linkage between dehydroalanine and basic residue(s) in the consensus site of kinases is predominant over exogenous nucleophilic groups. Interestingly, our data suggest also that the intramolecular linkage makes the basic residue involved unrecognizable by trypsin. Furthermore, recurrent cleavage of the carboxy terminal amino acids of the tryptic fragments by carboxypeptidase Y should be prevented by such an intra-cyclic arrangement (see Fig. 1D). We think that collection of the higher molecular weight peptides generated by drastic proteolytic cleavage using these two enzymes, and systematic N-terminal analysis of the few residues presenting such intramolecular linkage, could provide sufficient information to identify phospho-proteins and sites of phosphorylation.

Enzymatic digestions of extracts treated with and without alkali were compared. We tested two strains that have opposite status in terms of Cam kinase II dependent phosphorylation: under the control of the heat shock promoter, *ala2* expresses a pseudo-substrate inhibitory to Cam kinase II, while *RQED* expresses a constitutively active Cam kinase II (independent of calcium and calmodulin) [Broughton et al., 1996]. Furthermore, the treatment of head extracts with drugs such as BrcAMP (an activator of PKA), phorbol ester (an activator of PKC) or calcium (an activator of Cam K) *plus* presence of phosphatase inhibitors significantly induced the formation of peptides resistant to enzymatic digestion (see Fig. 1D). These data were obtained by the dosage of peptides collected in the void volume of a Sephadex G-10 column (Fig. 1D). In parallel, peptides were analyzed in acrylamide gel (see Fig. 1E). We observed that the acrylamide gel band above the gel migration front corresponds to fragments resistant to proteolytic treatment.

On the other hand, the consensus sequence of the target sites for casein kinase II contains many acidic residues; this is unique. Carboxylic residues are poorly nucleophilic and a priori should not undergo a Michael reaction with (methyl)dehydroalanine. However, the hydrophobicity generated by (methyl)dehydroalanine along with adjacent hydrophobic residue(s) could generate pockets from which water is excluded, triggering ester formation with a carboxylic group. In the future, analysis of the acidic fragments generated by mixtures of exo-carboxypeptidases and endo-proteases that cleave at acidic residues (e.g., protease V8) should provide insights into whether a parallel approach is valuable. However, we noticed that acidic consensus sites only occur in a small minority of serine/threonine kinases, essentially casein kinase II. The approach described in this report applies to most of the known serine and/or threonine kinases, such as PKA, PKC, PKG, and Cam kinase II, under the control of second messengers.

TABLE III. Diagram for Michael Addition on Dehydroalanine



A: dehydroalanine generated from phospho-serine after alkali treatment; B and C: Michael addition creates two diastereoisomers.

STEREOISOMERS GENERATED BY MICHAEL ADDITION ON DEHYDROALANINE MIGHT BE RESISTANT TO CARBOXYPEPTIDASES

The double bond in dehydroalanine means that the asymmetric Cα is lost (Table III). When the Michael addition occurs to dehydroalanine peptides by nucleophilic attack, the addition products regenerate asymmetric carbon (see Table III). Alternatively, this also might suggest that one configuration is not cleaved by carboxypeptidase for steric hindrance reasons. This might explain the high level of resistant peptides when the dehydroalanine group was alkylated by cystamine or ethylamine (see Fig. 1C).

CONCLUSIONS

The complete analysis of phosphopeptides from an extract will be a major focus in proteomics in future. From nowadays it seems a utopian goal and indeed unrealistic project. We suggest that the side effects generated by alkaline hydrolysis of phosphate in sufficiently mild conditions so that the other amino acids are not affected (cysteine and/or methionine groups can be selectively protected by oxidation) might be used to design methodologies in order to capture peptides previously phosphorylated. The high through put technologies might help in future to constitute catalogs of phosphopeptides (and proteins they come from) if simple protocols allow us to isolate them. We think that the direct derivatization of blotted proteins from acrylamide gels and labeled peptides recovery from an isolated band could be and advance in the attempt to compare samples submitted to different conditions.

On the other hand, we emphasize that these protocols and data are not relevant to tyrosine kinases. Tyrosine phosphate is more resistant to alkali hydrolysis, and commercially available monoclonal antibodies are more efficient.

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