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ORIGINAL ARTICLE

Monitoring of phosphorylated peptides by radioactive assay and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

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Abstract Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is frequently used to monitor phosphorylated peptides or protein kinase activities. However, few reports have compared a radioactivity assay with MALDI-TOF-MS analysis. We analyzed the phosphorylation ratios of 23 peptide substrates for G protein-coupled receptor kinase 2 (GRK2) with different lengths and numbers of negatively charged amino acids by MALDI-TOF-MS. We then examined the correlations between the phosphorylation ratios determined by MALDI-TOF-MS and the radioactivity levels (counts per minute, CPM) determined using a radioactive assay. Using MALDI-TOF-MS, the phosphorylation ratios were greater in the negative mode than in the positive mode. The phosphorylation ratio measured in the negative mode was strongly correlated with the CPM ($r = 0.86$). The number of acidic amino acids was related to the phosphorylation of peptide substrates by GRK2 ($r = 0.53$ and 0.46 for the phosphorylation ratio and CPM, respectively). These results suggest that MALDI-TOF-MS is an alternative to radioactive assays for monitoring phosphorylated peptides.

Keywords MALDI-TOF-MS · Negative mode · Phosphorylation · Protein kinase · Counts per minute · Radioactive assay

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Introduction

The phosphorylation of target proteins by protein kinases plays important roles in a variety of intracellular signal transduction pathways, including those involved in cellular differentiation, proliferation, migration, invasion, and apoptosis. In phosphorylation reactions, the amino acid sequences surrounding the phosphorylation site have an important effect on the phosphorylation of the target substrates. For example, protein kinase C (Kang et al. 2008b, 2012), protein kinase A (Smith et al. 2011), and Rho kinase (Kang et al. 2007a, 2011) show greater affinity for basic amino acids (e.g., arginine and lysine), whereas Iκ-B (Asai et al. 2009) and G protein-coupled receptor kinase 2 (GRK2) (Asai et al. 2014) show greater affinity for acidic amino acids (e.g., glutamate and aspartate).

Phosphorylated protein (or peptide) substrates are generally detected by radioactive assays using [^{32}P]-labeled ATP (Hastie et al. 2006) or nonradioactive assays, including enzyme-linked immunosorbent assays (Rykx et al. 2007), microarrays (Hoozemans et al. 2012; Kozlov et al. 2012), and mass spectrometry (MS) (Bowley et al. 2005).

Several MS-based assays, including high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS), are commonly used for the analysis of phosphorylated substrates. However, MALDI-TOF-MS is more efficient than HPLC–ESI-MS/MS in the analysis of small ions (<*m*/*z* 5000) (Irungu et al. 2008). MALDI-TOF-MS has several advantages over HPLC–ESI-MS/MS, including its accuracy, rapid measurement, simple procedure, high sensitivity, and the use of smaller samples (McLachlin and Chait 2001; Xu et al. 2005; Duncan et al. 2008). MALDI-TOF-MS is an efficient method for monitoring the peaks corresponding to phosphorylated peptides in phosphorylation experiments using purified enzymes (Kang et al. 2007a, 2011), or cell or tissue lysates (Kang et al. 2007b, 2008a, 2011). However, few reports have compared a radioactivity assay with MALDI-TOF-MS analysis.

In the present study, we synthesized 23 peptide substrates for GRK2 with different lengths and numbers of negatively charged amino acids. The phosphorylation ratios of these peptides were analyzed by MALDI-TOF-MS in the negative and positive modes. We then investigated the correlation between the phosphorylation ratios determined by MALDI-TOF-MS and the radioactivity levels determined using a radioactivity assay as counts per minute (CPM).

Materials and methods

Peptide synthesis

The peptide substrates were synthesized by standard Fmoc chemistry and were purified by HPLC. All peptides were modified by amino-terminal acetylation and carboxyl-terminal amidation. The molecular weight of each peptide was determined by MALDI-TOF-MS, as previously described (Kang et al. 2011). Peptides with a purity of >95 % were used as substrates for the phosphorylation reaction with GRK2 (Invitrogen, Carlsbad, CA, USA).

MALDI-TOF-MS analysis

The stock solution of synthetic peptides containing a final concentration 10 mg/ml was prepared in DMSO. The peptide solution for phosphorylation reaction was prepared by diluting a stock solution with 20 mM Tris–HCl at pH 7.5. The phosphorylation reaction for MALDI-TOF-MS analysis was carried out in 50 μl of buffer (20 mM Tris–HCl at pH 7.5, 100 μM ATP, and 10 mM $MgCl_2$) containing 30 μM peptide and 0.1 mg/ml of GRK2. After incubation for 60 min at 37 °C, the sample was analyzed by MALDI-TOF MS. The α-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml) was prepared in 50 % water/acetonitrile and 0.1 % trifluoroacetic acid. The α-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml) was prepared in 50 % water/acetonitrile and 0.1 % trifluoroacetic acid. The CHCA matrix and sample were mixed in 1:1 ratios. The matrix (20 μl) was added to a clean 1.5 ml tube before sample (20 μl) to prevent sample from adhering to the tube. The solution was mixed by vortexing at room temperature for 15–20 s. One microliter of the analyte/matrix mixture was applied to the Opti-TOF 384-well MALDI plate, which was dried at ambient temperature to induce crystallization.

All experiments were conducted using an AB SCIEX TOF/TOF 5800 (Applied Biosystems, Framingham, MA, USA) in the positive or negative modes. Prior to analysis, calibration was performed using a ProteoMass™ Peptide and Protein MALDI-MS Calibration Kit (Sigma-Aldrich, St. Louis, MO, USA), and the calibration mass errors were within ±0.5 *m*/*z*. Typically, 400 laser shots were averaged to improve the signal-to-noise ratio. All spectra were analyzed using Data Explore software (Applied Biosystems). The phosphorylation ratio for each peptide was calculated by the formula, [phosphorylated peptide/(phosphorylated peptide + unphosphorylated peptide) × 100] (Kang et al. 2007a).

Radioactive assay

The synthetic peptides were dissolved in dimethyl sulfoxide (DMSO) and final concentration of DMSO did not exceed 5 %. The kinase activity of recombinant GRK2 towards each peptide was determined by measuring ^{32}P transfer from [γ-^{32}P]ATP (6000 Ci/mmol, PerkinElmer Inc., Boston, MA, USA) to each peptide. Reagents were used according to the manufacturer's instructions. Phosphorylation reactions were carried out in 25 μl of buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM Na_3VO_4, 5 mM β-glycerophosphate, 2.5 mM dithiothreitol, 0.01 % Triton X-100, 200 μM ATP, and 0.1 μCi [γ-^{32}P]ATP) containing 50 μM peptide, and GRK2 (2.5 ng/μl). The assay mixture was incubated for 60 min at 30 °C, and the reaction was terminated by the addition of 5 μl of 30 % trichloroacetic acid (TCA). The reaction mixture (24 μl) was spotted onto polyvinylidene difluoride (PVDF) transfer membranes (Hybond-P, Amersham, 2 cm × 2 cm) that had been prewetted with methanol prior to equilibration in water (Asai et al. 2009). The PVDF membranes were washed three times with 5 % TCA, and the radioactivity on each membrane was determined by liquid scintillation counting.

Statistical analysis

The phosphorylation reactions were performed in triplicate and each sample was analyzed twice. The means,

standard deviations, and Pearson's correlation coefficients (r) between MALDI-TOF-MS data and CPM were calculated using Microsoft Excel Data Analysis (Microsoft, Redmond, VA, USA).

Results and discussion

Twenty-three peptide substrates used in this study were derived from known GRK2 substrates (#1–17), derivatives of peptide #1 (#18–22), and a standard peptide (RESA peptide, #23) (Table 1). The known GRK2 substrates (#1–17) are β2-adrenergic receptor (β2-AR) (Fredericks et al. 1996), β-tubulin (Yoshida et al. 2003), synucleins (α and β) (Pronin et al. 2000), Nedd4 (Sanchez-Perez et al. 2007), Nedd4-2 (Sanchez-Perez et al. 2007), epithelial Na^+ channels (ENaC) (Dinudom et al. 2004), phosducin (Ruiz-Gómez et al. 2000), rhodopsin (Ohguro et al. 1994), ribosomal protein P2 (Freeman et al. 2002), downstream regulatory element antagonist modulator (DREAM) (Ruiz-Gómez et al. 2007), p38 mitogen-activated protein kinase (p38 MAPK) (Peregrin et al. 2006), phosphodiesterase γ

Table 1 Peptide substrate sequences and number of acidic amino acids

Peptide #	GRK2 substrates	Peptide sequence [a] ←Amino terminus																		Carboxyl terminus→	Number of acidic amino acids [b]
		−9	−8	−7	−6	−5	−4	−3	−2	−1	***0***	+1	+2	+3	+4	+5	+6	+7	+8		
1	β-tubulin					D	E	M	E	F	***T***	E	A	E	S	N	M	N			5
2	β-tubulin					M	N	D	L	V	***S***	E	Y	Q	Q	Y	Q				2
3	β-synuclein				M	E	P	E	G	E	***S***	Y	E	D	P	P	Q				5
4	α-synuclein				E	A	Y	E	M	P	***S***	E	E	G	Y	Q	D				5
5	Nedd4-2				G	P	E	P	W	E	***T***	I	S	E	E	M	N	M			4
6	Nedd4				P	G	W	E	E	R	***T***	H	T	D	G	R	V	F			0
7	β2-AR			V	P	S	D	N	I	D	***S***	Q	G	R	N	C					1
8	ENaC			L	D	T	M	E	S	D	***S***	E	V	E	A	I					5
9	Phosducin					A	G	D	V	E	***S***	F	L	N	E	A					3
10	Rhodopsin				V	S	K	T	E	T	***S***	Q	V	A	P	A					0
11	Ribosomal protein P2				D	E	K	K	E	E	***S***	E	E	S	D	D	D	M	G		7
12	DREAM					K	K	E	L	Q	***S***	L	Y	R	G	F	K				−3
13	p38 MAPK				V	K	C	Q	K	L	***T***	D	D	H	V	Q	F				−1
14	PDEγ				G	M	E	G	L	G	***T***	D	I	T	V	I					2
15	PDGFR-β			P	R	A	E	A	E	D	***S***	F	L								2
16	Smad2				P	P	L	D	D	Y	***T***	H	S	I	P	E	N				2
17	Ezrin/radixin				G	R	D	K	Y	K	***T***	L	R	Q	I	R	Q	G			−4
18	Derivative of #1	G	E	G	M	D	E	M	E	F	***T***	E	A	E	S	N	M	N			6
19	Derivative of #1				M	D	E	M	E	F	***T***	E	A	E	S	N	M	N			5
20	Derivative of #1					D	E	M	E	F	***T***	E	A	E	S	N					5
21	Derivative of #1						E	M	E	F	***T***	E	A	E	S	N	M	N			4
22	Derivative of #1						E	M	E	F	***T***	E	A	E	S	N					4
23	RESA		R	R	R	E	E	E	E	E	***S***	A	A	A							2

A alanine, *C* cysteine, *D* aspartate, *E* glutamate, *F* phenylalanine, *G* glycine, *H* histidine, *I* isoleucine, *K* lysine, *L* leucine, *M* methionine, *N* asparagine, *P* proline, *Q* glutamine, *R* arginine, *S* serine, *T* threonine, *Y* tyrosine, *V* valine, *W* tryptophan

[a] The phosphorylation sites are indicated by bold italic font

[b] The number of acidic amino acids was calculated as the number of acidic amino acids − the number of basic amino acids

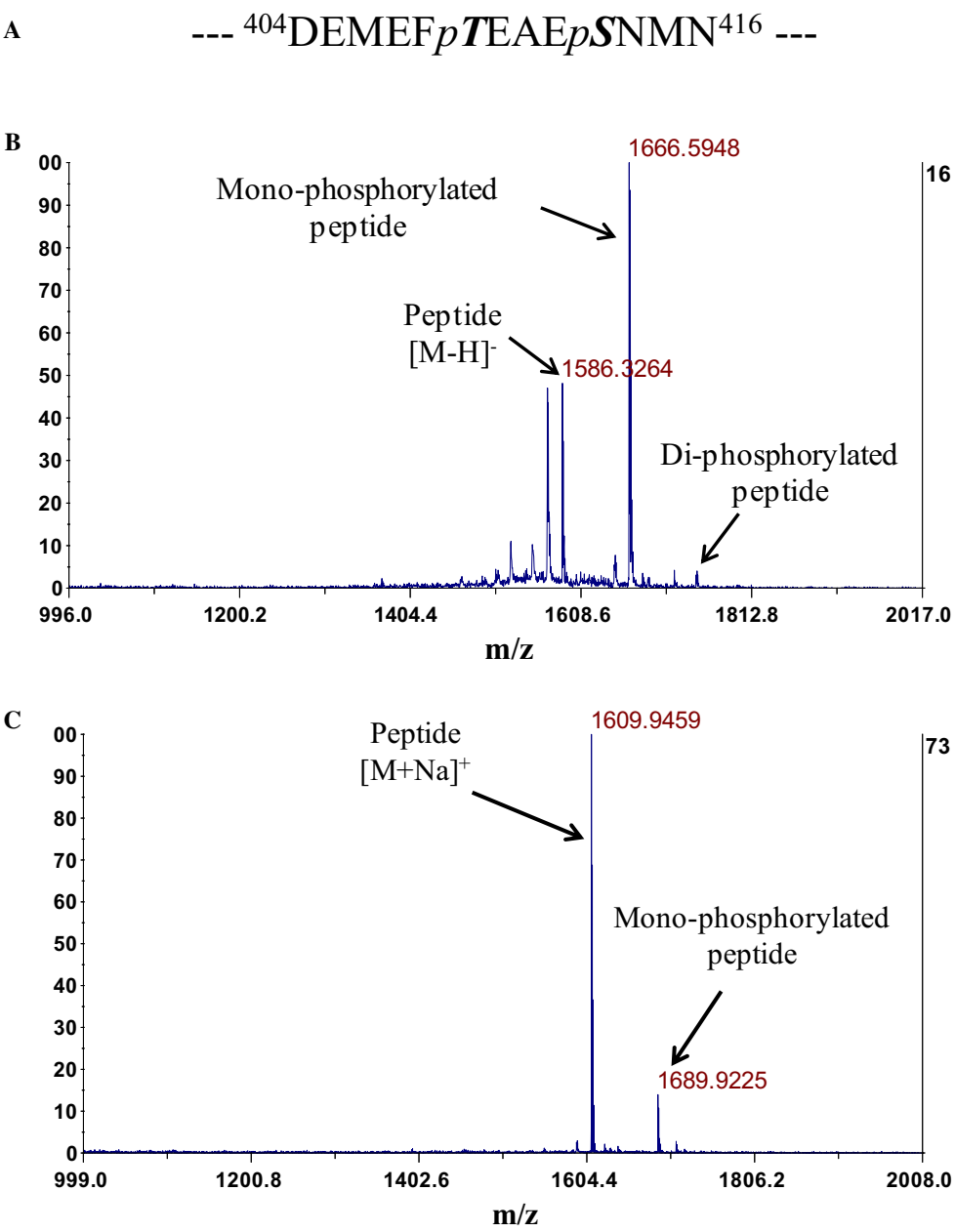

Fig. 1 Typical MALDI-TOF-MS spectra following the phosphorylation of peptide #1 by GRK2. **a** Sequence of peptide #1 modified by amino-terminal acetylation and carboxyl-terminal amidation. The peptide #1 contains two phosphorylation sites, Thr-409 and Ser-413. Peaks corresponding to phosphorylated peptides were detected by MALDI-TOF-MS in the negative mode (**b**) and in the positive mode (**c**). Phosphorylation increased the peak by 80 Da

(PDEγ) (Wan et al. 2001), Smad2 (Ho et al. 2005), ezrin/radixin (Cant and Pitcher 2005; Kahsai et al. 2010), and platelet-derived growth factor receptor-β (PDGFR-β) (Hildreth et al. 2004).

MALDI-TOF-MS detected peaks corresponding to phosphorylated peptides after each phosphorylation reaction. Generally, the phosphorylated peptides showed an increase in the *m/z* value of 80 Da compared with the unphosphorylated peptides (Table S1). The mass spectra for peptide #1 had peaks corresponding to mono- and di-phosphorylated peptides in the negative mode, but the peak corresponding to the di-phosphorylated peptide was not detected in the positive mode (Fig. 1). Peptide #1 was derived from β-tubulin and contains two phosphorylation sites, Thr-409 and Ser-413 (Yoshida et al. 2003). An earlier study revealed that Ser-413 is a poor phosphorylation target for GRK2 (Yoshida et al. 2003).

In the negative mode, peaks corresponding to phosphorylated peptide were detected in 13/23 peptides tested. The phosphorylation ratios determined in the positive mode were lower than those in the negative mode (Fig. 2). These results were similar to those of previous reports, which showed that the phosphorylated peaks of negatively charged peptides had greater signal intensities in the negative mode than in the positive mode (Janek et al. 2001; Ma et al. 2001; Kang et al. 2008c).

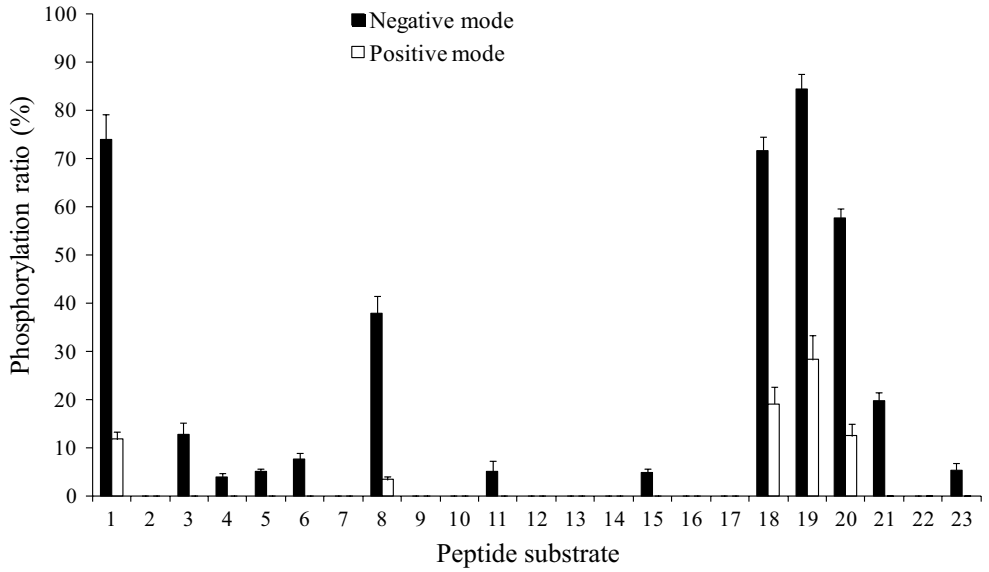

Fig. 2 Phosphorylation ratios for each peptide substrate. After phosphorylation of each peptide substrate with GRK2, peaks corresponding to phosphorylated peptides were detected by MALDI-TOF-MS in the negative mode and in the positive mode

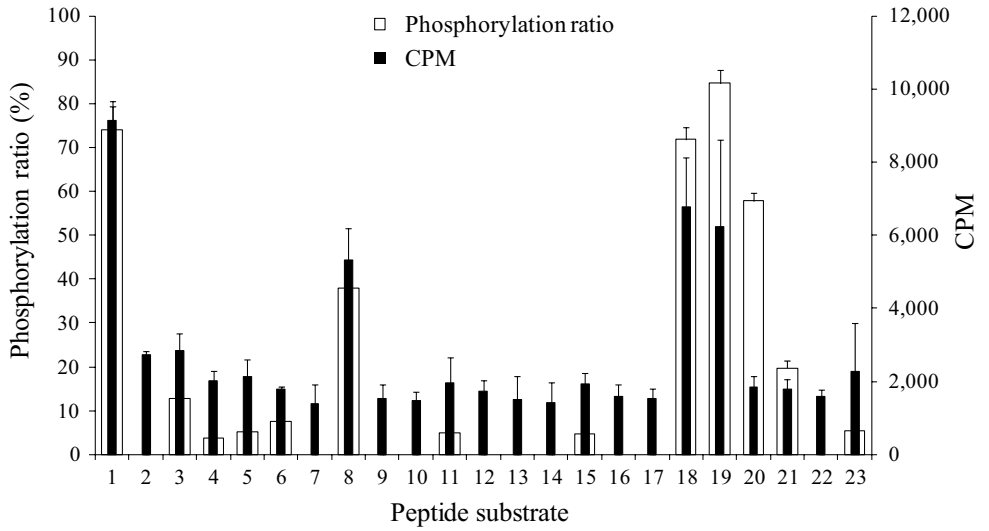

Fig. 3 Correlation between the phosphorylation ratio and the CPM for the peptide substrates. The phosphorylation ratios were determined by MALDI-TOF-MS in the negative mode and CPM data were taken from a previous study (Asai et al. 2014)

The CPM and phosphorylation ratios determined in the negative mode for 23 peptides are presented in Fig. 3. GRK2 offers a potential therapeutic target for various brain, tumor, inflammatory, and cardiovascular diseases, because of its hyperactivation in these diseases (Gainetdinov et al. 2004; Penela et al. 2010; Woodall et al. 2014). Highly specific and sensitive peptide substrates for GRK2 are useful for understanding the diversity of intracellular signaling pathways regulated by GRK2, and for developing GRK2-specific inhibitors. Although the peptide #1 displays both higher CPM and phosphorylation ratio than other GRK2 substrates (#2–17 and RESA peptide) (Fig. 3), a previous study has reported its relatively high specificity (K_m = 33.9 μM) but low sensitivity for GRK2 (V_{max} = 0.35 pmol/min/mg) (Asai et al. 2014). Therefore, further studies on the development of highly sensitive peptide substrates for GRK2 are needed.

The number of acidic amino acids was correlated with the phosphorylation of the peptides by GRK2 in terms of the phosphorylation ratio (r = 0.53) and CPM (r = 0.46)

Table 2 Correlation coefficients for all peptides and subgroups of peptides

Group	*r*
Peptides #1–23	0.86[a]
Peptides #1–17 and 23[b]	0.99[a]
Phosphorylated peptides[c]	0.79[a]
Number of acidic amino acids[d]	0.53[e]
	0.46[f]

[a] Correlation coefficient between CPM and the phosphorylation ratio determined in the negative mode

[b] Peptide substrates except for peptides derived from peptide #1

[c] Peptide substrates with phosphorylated peaks determined by MALDI-TOF-MS in the negative mode

[d] The number of acidic amino acids for each peptide was calculated as the number of acidic amino acids − the number of basic amino acids (see Table 1)

[e] Correlation coefficient between the phosphorylation ratio in the negative mode and the number of acidic amino acids

[f] Correlation coefficient between CPM and the number of acidic amino acids

(Table 2). Phosphorylated peaks were not detected in the negative mode for three positively charged peptides (#12, 13, and 17). GRK2 prefers peptides with acidic amino acids bordering the phosphorylation sites, as indicated by its consensus phosphorylation site motifs, such as (D/E)X_{1-3}(*S/T*), (D/E)X_{1-3}(*S/T*)(D/E), and (D/E)X_{0-2}(D/E)(*S/T*) (Asai et al. 2014). Thus, the limited phosphorylation of peptides containing few acidic amino acids may be due to the substrate specificity of GKR2.

Furthermore, the phosphorylation ratio determined by MALDI-TOF-MS was strongly correlated with the CPM ($r = 0.86$). A strong correlation between the phosphorylation ratio and the CPM was also found for the 13 peptide substrates that were phosphorylated by GRK2 ($r = 0.79$) (Table 2).

Previously, the phosphorylation ratio determined by MALDI-TOF-MS was reported to be correlated with enzyme kinetics (V_{max}/K_m) (Kang et al. 2008c). In the present study, we observed a strong correlation between the phosphorylation ratio determined in the negative mode and the CPM for negatively charged peptides. These results indicate that MALDI-TOF-MS is an alternative to radioactive assays for monitoring phosphorylated peptides.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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