Reports

Tools to discriminate between targets of CK2 vs PLK2/PLK3 acidophilic kinases

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While the great majority of Ser/Thr protein kinases are basophilic or proline directed, a tiny minority is acidophilic. The most striking example of such “acidophilic” kinases is CK2, whose sites are specified by numerous acidic residues surrounding the target one. However PLK2 and PLK3 kinases recognize an acidic consensus similar to CK2 when tested on peptide libraries. Here we describe optimal buffer conditions for PLK2 and 3 kinase activity assays and tools such as using GTP as a phosphate donor and the specific inhibitors CX-4945 and BI 2536, useful to discriminate between acidic phosphosites generated either by CK2 or by PLK2/PLK3.

BioTechniques 52:1-5 (July 2012) doi 10.2144/000113866

Keywords: acidophilic kinase; CK2; PLK2; PLK3

Over the past few years, research in the field of proteomics and phosphoproteomics has witnessed a tremendous revolution allowing for the highly confident characterization of protein phosphorylation on a global scale. Presently, more than 150,000 non-redundant phosphorylation sites have been identified from ~18,000 proteins (www.phosphosite.org). However, to date, the vast majority of phosphorylation events are still functionally uncharacterized and the link between the phosphorylation site and the kinases responsible is generally missing. In a previous report, we suggested that major contributions to the human phosphoproteome are provided by relatively few classes of protein kinases, with special reference to proline directed kinases, a few phosphate directed kinases whose targeting is primed by previously phosphorylated residues, and by the highly acidophilic protein kinase CK2 (1). In particular, we suggested that this latter kinase might be responsible alone for the generation of a substantial proportion of the eukaryotic phosphoproteome (about 20%), based on its consensus sequence, which shows distinctive features not shared by any other kinases (2). The minimum

consensus sequence of CK2 requires an acidic residue in position +3 downstream from the phosphoacceptor site (S/T-x-x-D/E/pS), and is generally accompanied by additional acidic residues (on average more than 5), being also characterized by the absence of basic residues in the proximity of the target aminoacid (2–4). Considering that the continuously growing repertoire of bona fide CK2 substrates already included more than 300 proteins in 2003 (4), CK2 could indeed represent the most pleiotropic member of the kinome.

Such a highly acidophilic consensus sequence was considered an unique signature of protein kinase CK2 until the recent discovery that two members of the Polo-like kinase (PLK) family, PLK2 and PLK3 display a strong preference for acidic residues at all positions between -4 and +4 with respect to the target aminoacid as judged from a peptide library assay (5). Moreover, the very limited number of bona fide PLK2 and PLK3 phosphosites identified so far confirms their highly acidophilic nature (see www.phosphosite.org for an updated list of identified phosphosites) (6). The preference of PLK2 and PLK3 for acidic side chains surrounding

the phosphoacceptor residue discloses the possibility that the consensus of these kinases could at least partially overlap that of protein kinase CK2. Indeed, as shown in Table 1, the consensus sequence extracted from peptide libraries for PLK2 and PLK3 is almost identical to the one calculated by Songyang et al. (7) for CK2 using an oriented peptide library. Accordingly, both CK2 and PLK2/PLK3 are assayed using the common artificial substrate casein.

These observations prompted us to develop tools to discriminate between phosphorylation performed by either CK2 or Polo-like acidophilic kinases.

Materials and methods

c-DNA constructs

Human PLK2-PGEX4TI (8), human CK2 α -PGEX4TI (9), and pcDNA3.1-CHMP3 (10) were previously described. For the preparation of the PLK3-PGEX4TI plasmid, the human c-DNA encoding full-length kinase inserted in pCMV6-XL4 vector was purchased from Origene (NM_004073) and amplified by PCR using primers to add *Bam*HI and *Xho*II restriction sites and inserted into the pGEX4TI vector

Method summary:

Here we describe optimal buffer conditions for PLK2 and PLK3 kinase assays and approaches to discriminate between targets of PLK2/PLK3 and CK2 acidophilic kinases, specifically the combined use of CX-4945 and BI 2536 as inhibitors in conjunction with GTP as a phosphate donor.

at *Bam*HI and *Xho*I sites.

The CHMP3S200A mutant was generated using the QuickChange-Site directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Mutation was confirmed by sequencing analysis.

Inhibitors

BI 2536 was purchased from Selleck Chemicals (Houston, TX, USA), CX-4945 was purchased from Synthesis Medchem (Cambridge, UK), 4,5,6,7-tetrabromobenzotriazole (TBB) was synthesized as described in Sarno et al. (11).

Expression and purification of recombinant kinases

Expression and purification of GST-CK2 α , GST-PLK2, and GST-PLK3 was performed as described in our earlier published work (8).

In vitro phosphorylation

Reaction conditions for α -casein phosphorylation experiments were the following: 50 mM Tris/HCl, pH 7.5, 50 μ M, (γ - 33 P) ATP (specific radioactivity $\sim$ 3000 cpm/pmol), 1.5 μ g α -casein, with the addition of different amounts of MgCl $_2$, NaCl, or DTT (20 μ l final volume). This amount of casein corresponds to the apparent Km of the phosphorylation reaction by PLK2 and PLK3 calculated in preliminary experiments (not shown). Experiments with inhibitors were performed with 5 μ M (γ - 33 P)ATP (specific radioactivity $\sim$ 3000 cpm/pmol) in order to be at the Km for ATP for the kinases (Km for ATP for PLK2, PLK3, and CK2 is between 5–6 μ M, as calculated in preliminary experiments not shown). The reaction was started by the addition of protein kinases (50 ng PLK2, 20 ng PLK3, 10 ng CK2). The reaction mixtures were incubated for 10 min at 30°C and stopped by the addition of Laemmli buffer and boiling followed by SDS-PAGE and Coomassie staining. Gels were dried and exposed overnight to a multipurpose storage phosphor screen and analyzed using a Cyclone storage phosphor system (Perkin Elmer, Massachusetts, USA).

Cell culture, transfection, and immunoprecipitation

293T cells were maintained in 5% CO $_2$ in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 mM streptomycin in an atmosphere containing 5% CO $_2$. DNA Transfection was performed with Trans-IT (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Forty-eight

Table 1. Position relative to phosphoacceptor residue.

	-4	-3	-2	-1	0	+1	+2	+3	+4
PLK2	E	E/D	X	E/D	S/T	E/D	E/D	E/D	E/D
PLK3	E	E/D	E/D	E/D	S/T	E	E/D	E/D	E/D
CK2	E	D	E	E	S/T	E	D	E	E

Overlapping of consensus sequences derived from peptide libraries for protein kinases PLK2, PLK3 and CK2.

Figure 1. Characterization of PLK2 and PLK3 kinase activity on α -casein. (A–B) 1.5 μ g of α -casein was phosphorylated by PLK2 or PLK3 in a radioactive mixture (50 mM Tris, pH 7.5, 50 μ M (γ - 33 P)ATP, specific radioactivity $\sim$ 3000 cpm/pmol) in the presence or absence of increasing concentrations of Mg $^{2+}$, Mn $^{2+}$ (A) and NaCl (B) as indicated. Proteins were resolved by SDS/PAGE, Coomassie stained, and analyzed by PhosphorImager. A representative picture of three independent experiments is shown. On the right is reported the effect on α -casein activity of 5 and 10 mM Mn $^{2+}$ as a percentage of the activity measured with 10 mM Mg $^{2+}$ (A) and NaCl effect as a percentage of the activity measured in its absence (B) (mean values $\pm$ SD of three determinations are shown). (C) Phosphorylation of α -casein by PLK2, PLK3, and CK2 in the presence of ATP or GTP. 1.5 μ g of α -casein was phosphorylated by PLK2, PLK3, or CK2 in 50 mM Tris (pH 7.5), 10 mM Mg $^{2+}$, 50 μ M (γ - 33 P)ATP (specific radioactivity $\sim$ 3000 cpm/pmol) (lane 1), or 50 (lane 2) or 100 μ M (lane 3) (γ - 33 P)GTP (with the same specific radioactivity) (mean values $\pm$ SD of three determinations are shown). (D) 1.5 μ g of α -casein was phosphorylated by PLK2 or PLK3 in a radioactive mixture (50 mM Tris, pH 7.5, 10 mM Mg $^{2+}$, 50 μ M (γ - 33 P)ATP, specific radioactivity $\sim$ 3000 cpm/pmol) in the presence or absence of 1 mM DTT. Proteins were resolved by SDS/PAGE, Coomassie stained, and analyzed by PhosphorImager. A representative picture of three independent experiments is shown. On the right, the effect of DTT is quantified (mean values $\pm$ SD of three determinations are shown).

Figure 2. Effect of inhibitors on CK2 and PLK2/PLK3 kinase activity. Compared effect of TBB (A), CX-4945 (B), and BI 2536 (C) on PLK2, PLK3, and CK2 activity. 2 μ g of α -casein was phosphorylated by PLK2, PLK3, or CK2 in the presence of increasing concentrations of inhibitor (mean values $\pm$ SD of three determinations are shown) in 50 mM Tris (pH 7.5), 10 mM Mg^{2+} , 1 mM DTT, 50 μ M (γ - ^{33}P)ATP (specific radioactivity $\sim$ 3000 cpm/pmol). IC_{50} for TBB were 0.35 μ M (CK2), 0.65 μ M (PLK3), 0.84 μ M (PLK2). IC_{50} for CX-4945 are 0.03 μ M (CK2), 0.88 μ M (PLK3), 0.56 μ M (PLK2). IC_{50} for BI2536 are 0.08 μ M (PLK3), 0.07 μ M (PLK2) (not determined for CK2).

hours after transfection, cells were lysed in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 (v/v), Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, Indiana, USA). Lysates were incubated with monoclonal anti-cMyc antibody (clone 9E10; Sigma), then with 30 μ l of protein G-PLUS-Agarose (Santa Cruz Biotechnology). Beads were washed with PBS and incubated in the radioactive mixture consisting of 50 mM Tris (pH 7.5), 10 mM Mg^{2+} , and 50 μ M ATP (γ - ^{33}P)ATP $\sim$ 3000 cpm/pmol or 100 μ M GTP (γ - ^{33}P)GTP $\sim$ 3000 cpm/pmol, in presence or absence of specific inhibitors. The reaction was stopped with the addition of 2 $\times$ Laemmli sample buffer, subjected to SDS/PAGE, and blotted on PVDF. Blots were exposed overnight to a multipurpose storage phosphor screen and analyzed using a Cyclone storage phosphor system (Perkin Elmer).

In silico analysis

To analyze the molecular reasons for the cross-selectivity between the inhibitors BI 2536 and CX4945, the crystal structure complexes of BI 2536/PLK1 (PDB ID: 2RKU) and CX-4945/CK2 (PDB ID: 3PE1) were exploited. An in silico analysis was carried out through molecular docking experiments using MOE program (C. C. G. Molecular Operating Environment

(MOE 2009.10), Inc., 1255 University St., Suite 1600, Montreal, Quebec, Canada, H3B 3X3) and Schrödinger Glide (Glide, version 5.5, Schrödinger, Inc., New York, NY, 2009). Human PLK2/3 catalytic subunits were built using an homology modeling approach implemented into MOE, using the PLK1 crystal structure (2RKU) as a template. Hydrogen atoms were added to the protein structure using standard geometries with MOE; to minimize contacts between hydrogens, the structures were subjected to Amber99 force field minimization until the root mean square deviation of the conjugate gradient was <0.1 kcal $\cdot$ mol $^{-1}$ $\cdot$ Å $^{-1}$ (1 kcal = 4.184 kJ; 1 Å = 0.1 nm), keeping the heavy atoms fixed at their crystallographic positions. BI 2536 and CX4945 were rebuilt using MOE builder and minimized using PM3 semi-empirical quantum mechanics force field implemented in Mopac 7. A set of docking runs were performed using the program Glide.

Results and discussion

We determined the optimal incubation conditions and cofactor requirements for PLK2 and PLK3 kinase activity. These details have not previously been provided for these two kinases, but the conditions are well established for CK2 (12, 13). Here, we used α -casein (Figure 1) as an artificial

phosphorylatable substrate (equally suited for testing CK2) and repeated the experiments with the physiological PLK2/PLK3 substrate, α -synuclein. We obtained comparable results from both substrates (Figure S1). Figure 1A shows that both kinases prefer Mg^{2+} over Mn^{2+} , the latter being almost ineffective with PLK3. In particular, our results show that 2–10 mM Mn^{2+} could be also used for PLK2 kinase assay (with a reduction of efficiency of $\sim$ 40%–50% if compared with the activity obtained with 10 mM Mg^{2+}). Conversely, PLK3 activity obtained with all the Mn^{2+} concentrations tested is always less than 10% of that obtained with 10 mM Mg^{2+} (Figure 1A). NaCl exerts a dose-dependent inhibitory effect on both kinase activities, with stronger effects on PLK3 activity (Figure 1B). The use of 10 mM Mg^{2+} without NaCl is therefore suggested. This is the same as commonly used for protein kinase CK2 (12, 13).

A peculiar characteristic of protein kinase CK2 is its ability to use GTP as a phosphodonor substrate almost as efficiently as ATP (13). This property has been utilized to highlight the CK2-dependent phosphoproteome in cellular lysates (14). In Figure 1C, we compared the ability of PLK2, PLK3, and CK2 to use either ATP or GTP in the phosphorylation reaction. As expected, GTP can effectively replace ATP for CK2 activity, but this was not the case for PLK2 and PLK3. Therefore using GTP allows discrimination between the activity of CK2 and PLK2/PLK3 kinases. Figure 1D analyzes the possibility that PLK2 and PLK3 are redox-regulated enzymes: the addition of the reducing agent DTT shows a strong increase (up to six times) of α -casein phosphorylation by both kinases (Figure 1D). In silico analysis for cysteines that potentially can form intra or intermolecular disulfide bridge and that are conserved in both PLK2 and PLK3 kinases disclosed two cysteines located in the ATP binding cleft (Cys 96 and 162 for PLK2; Cys 76 and 142 for PLK3) and one in the activation loop (PLK2 Cys 241, PLK3 Cys 221) (Figure S2). Intriguingly these cysteines are conserved also in PLK1 (Cys 67, Cys 133, and Cys 212), highlighting the possibility that this group of kinases presents a similar mechanism of redox regulation.

Therefore, optimal buffer conditions for both PLK2 and PLK3 kinases are 50 mM Tris pH 7.5, 10 mM Mg^{2+} , 1 mM DTT, and no NaCl. This buffer is the same as utilized for CK2, except for the presence of DTT since the activity of this kinase is insensitive to reducing agents (15).

Specific inhibitors could represent

we focused our attention on CHMP3, a protein of the ESCRT-III complex that is required for multivesicular body (MVB) formation (19). CHMP3 phospho S200, identified in different cell lines in large scale mass spectrometry experiments (see www.phosphosite.org for references), is adjacent to a very acidic cluster (PPGAMAApSEDEEEEE) and the kinase responsible has not been identified. As shown in Figure 4, CHMP3 immunoprecipitated from 293T cells is phosphorylated by a kinase that coimmunoprecipitates with the protein. To gain information about the kinase responsible for CHMP3 S200 phosphorylation, use of GTP (instead of ATP) as a phosphate donor and inhibitors BI 2536 and CX-4945 was exploited. The results shown in Figure 4 show that GTP is able to replace ATP in the phosphorylation reaction and that phosphorylation is completely refractory to high concentrations of BI 2536, while potently inhibited by a low concentration of CX-4945. Note that phosphorylation of CHMP3 is completely abrogated by the mutation of a phospho-acceptor residue (S200), which has been shown to be phosphorylated in vivo (see inset in Figure 4). Taken together, these results provide a clear-cut demonstration that the kinase that coimmunoprecipitates with and phosphorylates CHMP3 is CK2, not PLK2/PLK3, suggesting that CK2 is also the kinase responsible for CHMP3 phosphorylation in vivo.

In conclusion, the combined use of CX-4945 and BI 2536, in conjunction with GTP as phosphate donor, allows a neat discrimination between acidic phosphosites generated either by CK2 or by PLK2/PLK3.

Acknowledgments

We thank The Molecular Modeling Section (MMS), coordinated by Professor S. Moro (Padova, Italy). This work was supported by University of Padova Progetto Ateneo CPDA089591/08 to M.S. and by Associazione Italiana per la Ricerca sul Cancro (AIRC) to L.A.P.

Competing interests

The authors declare no competing interests.

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Received 24 April 2012; accepted 22 June 2012.

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