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 PLK3 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.

Keywords: acidophilic kinase; CK2; PLK2; PLK3

Materials and methods
c-DNA constructs
Human PLK2-PGEX4T1 (8), human CK2α-PGEX4T1 (9), and pcDNA3.1-CHMP3 (10) were previously described. For the preparation of the PLK3-PGEX4T1 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 BamHI and XhoI restriction sites and inserted into the pGEX4T1 vector.

Method summary:
Here we describe optimal buffer conditions for PLK2 and PLK3 kinase assays and approaches to discriminate between targets of PLK2/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.
at BamHI and XhoI 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-tetramethylbenzozotriazole (TBB) was synthesized as described in Sarno et al. (11).

Expression and purification of recombinant kinases
Expression and purification of GST-CK2α, 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 µM (γ-33P)ATP (specific radioactivity ~3000 cpm/pmol), 1.5 µg α-casein, with the addition of different amounts of MgCl2, NaCl, or DTT (20 µl 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 µM (γ-33P)ATP (specific radioactivity ~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 µM, 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 by Cyclone storage phosphor system (Perkin Elmer, Massachusetts, USA).

Cell culture, transfection, and immunoprecipitation
293T cells were maintained in 5% CO2 in DMEM supplemented with 10% FBS, 2 mM1-glutamine, 100 U/mL penicillin, and 100 mM streptomycin in an atmosphere containing 5% CO2. DNA Transfection was performed with TransIT (Mirus, Madison, WI, USA) according to the manufacturer’s instructions. Forty-eight

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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 µg of α-casein was phosphorylated by PLK2 or PLK3 in a radioactive mixture (50 mM Tris, pH 7.5, 50 µM (γ-33P)ATP, specific radioactivity ~3000 cpm/pmol) in the presence or absence of increasing concentrations of Mg2+, Mn2+ (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 PLK2 and PLK3 activity of 5 and 10 mM Mn2+ as a percentage of the activity measured with 10 mM Mg2+ (A) and NaCl effect as a percentage of the activity measured in its absence (B) (mean values ± 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 µg of α-casein was phosphorylated by PLK2, PLK3, or CK2 in 50 mM Tris (pH 7.5), 10 mM Mg2+, 50 µM (γ-33P)ATP (specific radioactivity ~3000 cpm/pmol) (lane 1), or 50 (lane 2) or 100 µM (lane 3) (γ-33P)GTP (with the same specific radioactivity) (mean values ± SD of three determinations are shown). (D) 1.5 µg of α-casein was phosphorylated by PLK2 or PLK3 in a radioactive mixture (50 mM Tris, pH 7.5, 10 mM Mg2+, 50 µM (γ-33P)ATP, specific radioactivity ~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 ± SD of three determinations are shown).
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 µl of protein G-PLUS-Agarose (Santa Cruz Biotechnology). Beads were washed with PBS and incubated in the radio-active mixture consisting of 50 mM Tris (pH 7.5), 10 mM Mg²⁺, and 50 µM ATP ([γ-³²P]ATP ~3000 cpm/pmol) or 100 µM GTP ([γ-³²P]GTP ~3000 cpm/pmol), in presence or absence of specific inhibitors. The reaction was stopped with the addition of 2 × 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 hydrogenes, the structures were subjected to Amber99 force field minimization until the root mean square deviation of the conjugate gradient was <0.1 kcal • mol⁻¹•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 Mopac7. 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 Mg²⁺ over Mn²⁺, the latter being almost ineffective with PLK3. In particular, our results show that 2–10 mM Mn²⁺ could be also used for PLK2 kinase assay (with a reduction of efficiency of ~40–50% if compared with the activity obtained with 10 mM Mg²⁺). Conversely, PLK3 activity obtained with all the Mn²⁺ concentrations tested is always less than 10% of that obtained with 10 mM Mg²⁺ (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 Mg²⁺ 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 (15). 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 inter-molecular disulfide bridge and that are conserved in both PLK2 and PLK3 kinases disclosed two cysteines located in the ATP binding cleft (Cys 96 and 142 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 Mg²⁺, 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...
useful tools to discriminate between targets affected by either CK2 or PLK2/PLK3. Therefore CK2 inhibitor 4,5,6,7-tetra-bromo-1H-benzotriazole (TBB) and the recently developed CK2 inhibitor CX-4945, which is in Phase I clinical trials for treating cancer (16), and BI 2536, a potent inhibitor of PLK kinases (17) were compared for their ability to inhibit CK2, PLK2, and PLK3. TBB is the most widely used CK2 inhibitor and its selectivity has been previously tested at 1 and 10 µM on a panel of 70 kinases which didn’t include PLK2/PLK3 kinases (18). Selectivity of CX-4945 has been tested at a single concentration on a panel of 235 kinases, including PLK2 and PLK3, which were only slightly inhibited (≤10% at 0.5 µM) (16). Figures 2A and 2B show the effect of TBB and CX-4945 on kinase activities. TBB was not useful in discriminating between CK2 and PLK since PLK2 and PLK3 were inhibited with a similar IC_{50} (Figure 2A). In contrast CX-4945 is almost ineffective on PLK2 and PLK3 up to 200 nM concentration while CK2 at this concentration is totally inhibited (Figure 2B). Figure 2C shows the effects of PLK inhibitor BI 2536. The selectivity of this compound against CK2 has never been tested. BI 2536 is absolutely specific, giving clear-cut results: CK2 is fully refractory to its inhibition up to concentrations of 10 µM (not shown), while PLK2 and PLK3 activities are completely suppressed by sub-micromolar concentrations of BI 2536. The structural basis for the cross-selectivity between the two inhibitors could be explained by in silico analysis. The binding mode of BI 2536 is conserved both in PLK2 and PLK3; the sequence similarity of the three polo-like kinases at 4.5Å from BI 2536 is about 80% and all the residues interacting with BI 2536 are conserved. On the other hand, the PLK and CK2 ATP binding clefts are very different (Figure 3A); the CK2 cavity is more hydrophobic and smaller, due to bulkier residues. PLK2/3 present Ala 109/89, Leu 159/139, Cys 96/76, Leu 88/68, Cys 162/142, while in CK2 the corresponding residues are Val 66, Phe 113, Val 51, Leu 59, Val116. For this reason, in the CK2 active site there is not enough space to accommodate BI 2536; in particular CK2 Val 66 and Val 53 fill up the space for of BI 2536 7R-ethyl group, thus preventing the binding of the inhibitor to the hinge region.

On the other hand, the selective CK2 inhibitor CX-4945 binds to the PLK2/3 active site much less efficiently, since a point mutation study on CK2 has revealed that the inhibitor binding is largely dependent on hydrophobic residues, notably V66 and I174 (16), which are replaced in PLK2/3 by smaller and less hydrophobic residues. Moreover, PLK2/3 cannot host the stabilizing water molecule due to the presence of Arg 165/145 instead of Asn 118 in CK2 (Figure 3B).

Collectively, these results indicate that the combined use of CX-4945 and BI 2536 effectively discriminate between CK2 and PLK2/PLK3.

To probe the reliability of this approach,
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 (PGAMAAPSEDEEEEE) 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 abrogated by the mutation of a phospho-acceptor residue (S200), which has been

Acknowledgments

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Competing interests

The authors declare no competing interests.

References