A Generic Time-resolved Fluorescence Assay for Serine/threonine Kinase Activity: Application to Cdc7/Dbf4

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The serine/threonine protein kinase family is a large and diverse group of enzymes that are involved in the regulation of multiple cellular pathways. Elevated kinase activity has been implicated in many diseases and frequently targeted for the development of pharmacological inhibitors. Therefore, non-radioactive antibody-based kinase assays that allow high throughput screening of compound libraries have been developed. However, they require a generation of antibodies against the phosphorylated form of a specific substrate. We report here a time-resolved fluorescence assay platform that utilizes a commercially-available generic anti-phospho-threonine antibody and permits assaying kinases that are able to phosphorylate threonine residues on protein substrates. Using this approach, we developed an assay for Cdc7/Dbf4 kinase activity, determined the $K_m$ for ATP, and identified rottlerin as a non-ATP competitive inhibitor of this enzyme.

Keywords: Cdc7, MCM2, Rottlerin, Serine/threonine kinase, Time-resolved fluorescence

Introduction

Serine and/or threonine phosphorylation is a ubiquitous mechanism for protein regulation that is found in multiple signaling pathways in eukaryotic cells (Hunter, 1995). It is catalyzed by serine/threonine protein kinases, a large and diverse family of enzymes that are involved in the regulation of cell proliferation, differentiation, and metabolism (Hunter, 2000). In recent years, a growing number of serine/threonine kinases have been implicated in the development and progression of human diseases, and have been proposed as therapeutic targets. Accordingly, non-radioactive kinase assays that allow high throughput screening of compound libraries have been developed and used for the identification of kinase inhibitors (Gaarde et al., 1997; Mallon et al., 2001).

Among the most widely used ones today are assays that measure the time-resolved fluorescence signal from Europium chelates conjugated to antibodies that recognize phospho-serine or phospho-threonine residues on peptide substrates (Bader et al., 2001). These assays suffer from two main limitations: (a) They require a generation of specific antibodies against phospho-peptides that can serve as surrogate kinase substrates. (b) They generally do not permit the use of natural protein substrates. Recently, Cell Signaling Technology Inc. (CST, Beverly, USA) introduced a generic anti-phospho-threonine antibody that can recognize phospho-threonine in both proteins and peptides, regardless of their amino acid environment (CST, product no. 9381). Using this antibody, we developed a time-resolved fluorescence assay for Cdc7 kinase, that is generally applicable to many serine/threonine kinases that can phosphorylate threonine residues on protein or peptide substrates.

Cdc7 is a serine/threonine kinase that plays a critical role in the regulation of the initiation step of DNA replication in eukaryotic cells (Jiang et al., 1999; Masai and Arai, 2002). Similar to cyclin-dependent kinases, Cdc7 is activated at the G1 to S phase transition by a cyclin-like protein Dbf4. It is believed that activated Cdc7/Dbf4 phosphorylates the proteins of the MCM family, thus triggering the molecular events that are essential for the initiation of DNA synthesis at the origins of replication (Lei and Tye, 2001). Cdc7 is overexpressed in human tumors; therefore, its inhibition may offer a novel approach to cancer therapy (Hess et al., 1998). We report here a high throughput Cdc7 kinase assay that uses its natural protein substrate MCM2, and allows for the discrimination of ATP-competitive and non-competitive inhibitors. Using this assay, we determined the $K_m$ for ATP of Cdc7/Dbf4, and identified rottlerin as a Cdc7 inhibitor.

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Materials and Methods

Materials Anti-phospho-threonine antibody (CST, product no. 9381) was prepared by Cell Signaling Technology and labeled with Europium chelate (W1024) by PerkinElmer Wallac (Gaithersburg, USA) (product no. AD0092). The TRF (time-resolved fluorescence) assay buffer, wash buffer, and enhancement solution were purchased from PerkinElmer Wallac. The γ33P-ATP was from NEN (Boston, USA). SB 202190, KT 5720, and K-252C were purchased from Calbiochem (San Diego, USA). The anti-Cdc7 and anti-Dbf4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). All other chemicals were from Sigma (St. Louis, USA), unless otherwise indicated.

Cloning and expression Human Cdc7 and Dbf4 were amplified from a human testis cDNA library using PCR, and cloned into the SfiI sites of a modified pFASTBAC baculovirus expression vector. The Cdc7 and Dbf4 sequences were appended by an EE-tag (EFMPME) and FLAG-tag, respectively. The proteins were co-expressed in H5 cells and purified as a complex by immunopurification chromatography with an anti-EE-tag monoclonal antibody that was coupled to protein G-Sepharose (Stern and Podlaski, 1993). His-tagged human MCM2 (N-terminal 467 amino acids) was cloned, expressed in bacterial cells, and purified, as described elsewhere (Todorov et al., 1994).

Radioactive kinase assay The assay was carried out in a total volume of 250 µl that contained 5 µg/ml Cdc7/Dbf4, 60 µg/ml His6-MCM2, 4 µM ATP, 10 mM MgCl2, 1 mM DTT (dithiothreitol), 5 µg/ml leupeptin in 50 mM Hepes, pH 7.5, and 2.92 µCi γ33P-ATP (3000 Ci/mmol, Amersham, Piscataway, USA). After an 1 h-incubation at 37°C, 100 µl of the reaction volume was transferred to new vials, and the reaction was stopped by the addition of a 0.37 ml stop solution that contained 20 mM EDTA, 50 µM ATP, 0.5% BSA (bovine serum albumin), and 3.3 mg/ml SPA (scintillation proximity assay) beads that were coated with anti-His antibody (Amersham) in PBS (phosphate-buffered saline). After an additional 1 h-incubation at room temperature, the mixture was transferred to 96-well MultiScreen filter plates (Millipore, Bedford, USA) in duplicate wells. The beads were collected by vacuum filtration and washed 4 times with PBS that contained 0.1% Triton X100. The plates were sealed and counted in a Microbeta reader (PerkinElmer Wallac). The percent MCM2 phosphorylation was calculated by taking into account the specific activity of the γ33P-ATP and 60% counting efficiency.

TRF kinase assay High-binding 384-well plates (Costar #3708) were coated with 40 µl of His6-MCM2 in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) at room temperature for 1 h with shaking. They were washed once with TBS (Tris-buffered saline) that contained 0.01% Tween 20, blocked with 0.5% BSA in TBS for 2 h at room temperature, and washed three times with TBS/Tween 20. The enzyme reaction was started by adding Cdc7/Dbf4 and ATP in a total volume of 40 µl that contained 10 mM MgCl2, 1 mM DTT, 5 µg/ml leupeptin, and 0.02% BSA in 50 mM Hepes, pH 7.5. After a 30 min-incubation at room temperature, the plates were washed three times with a TBS/Tween 20 buffer and incubated with a 40 µl Eu-labeled anti-phospho-threonine antibody (0.16 µg/ml) in a TRF assay buffer at room temperature for 1 h. They were then washed 6 times with a TRF wash buffer, and the fluorescent signal was amplified by adding 40 µl enhancement solution. The TRF signal was measured by a Victor-5 plate reader (PerkinElmer Wallac) using excitation of 340 nm and emission of 615 nm (400 µs delay and 400 µs acquisition window).

Results and Discussion

Cdc7/Dbf4 were co-expressed in insect cells and purified as an equimolar complex with approximately a 60% purity. Their identity was confirmed by Western blotting using Cdc7 and Dbf4-specific antibodies (not shown). To confirm that the Cdc7/Dbf4 preparation was catalytically active on MCM2, we used a scintillation-proximity-based γ33P-ATP kinase assay. His6-MCM2 was captured on the surface of the SPA beads that were coated with the anti-His antibody and separated from the excess radiolabel by filtration through glass fiber membranes in a 96-well filter plate. After a 1 h-incubation of 60 µg/ml MCM2 with 5 µg/ml Cdc7/Dbf4 in the presence of 4 µM, ATP led to the phosphorylation of an estimated 11% of the MCM2 molecules, assuming a single phosphorylation event per substrate molecule (data not shown). This experiment indicated that our recombinant Cdc7/Dbf4 is catalytically competent and can utilize recombinant MCM2 as a substrate.

The rabbit polyclonal anti-phospho-threonine antibody that was developed by Cell Signaling Technologies can recognize phosphorylated threonine irrespective of its context in both of the small peptides or full length proteins (CST 9381, product insert). This property offers the possibility of designing non-radioactive kinase assays with generic applicability to the serine/threonine kinase family of enzymes. Using an Europium-conjugated version of the antibody, we developed a time-resolved fluorescence assay for the Cdc7/Dbf4 kinase activity. In this assay, the degree of phosphorylation is measured by the amount of the Eu-labeled anti-phospho-threonine antibody that is retained on MCM2-coated 384-well plates after extensive washing (see Materials and Methods).

The cross-titration of Cdc7/Dbf4 (0.6-2.4 µg/ml) and His6-MCM2 (0.125-8.0 µg/ml) showed that the fluorescence signal is dependent on both the enzyme and substrate concentrations (Fig. 1A, B) and reflects the phosphorylation status of MCM2. The low but detectable background signal that was observed in the absence of His6-MCM2 increased with the Cdc7/Dbf4 concentration. This indicates that it is most likely derived from the autophosphorylation of threonine residues on Cdc7/Dbf4 (Fig. 1A). This autophosphorylation was previously reported using the recombinant Cdc7/Dbf4 complex (Masai et al., 2000). The background was negligible at low Cdc7/Dbf4 concentrations that allowed a high signal to background ratio (25 : 1 at 0.6 µg/ml Cdc7/Dbf4 and 8.0 µg/ml His6-MCM2). To address the Dbf4 role in the activation of Cdc7, we expressed and purified the Cdc7 kinase in the absence of Dbf4. When Cdc7/Dbf4 was replaced with Cdc7...
alone, no signal was detected (data not shown). This confirmed previous observations that Dbf4 is essential for the activation of the Cdc7 kinase (Lei and Tye, 2000; Masai et al., 2000). The lack of a fluorescence signal in the absence of Dbf4 also indicated that the signal is generated by the activity of the Cdc7/Dbf4 complex, but not a contaminating kinase.

Next, we examined the ATP-binding kinetics of Cdc7/Dbf4. The standard kinase activity methods, based on radioactive ATP, did not allow \( K_m \) measurement. Therefore, electrophoretic separation of phosphorylated MCM2 has been used as a means of measuring the ATP affinity of this kinase (Masai et al., 2000). This semi-quantitative approach derived an apparent \( K_m \) value of 1-2 \( \mu M \). Our quantitative study, using a Michaelis-Menten plot, determined a \( K_m \) for ATP of 0.5 \( \mu M \) (Fig. 2). This \( K_m \) value is significantly lower than the \( K_m \) for most known representatives of the serine/threonine class, and puts Cdc7/Dbf4 in an unfavorable position for targeting with ATP-competitive inhibitors. However, the time-resolved fluorescence assay reported here tolerates high ATP concentrations, and permits the identification of non-ATP competitive inhibitors of this important kinase.

To validate the assay as a drug discovery tool, we looked for small-molecule inhibitors of Cdc7/Dbf4. Since there are no reported Cdc7 inhibitors, a panel of 12 compounds (rapamycin, quercetin, wortmannin, rottlerin, KN-62, SB203580, K-252C, H89, U0126, LY294002, PD98059, and KT5720) with previously-reported activity against a diverse
set of protein kinases (Davies et al., 2000) was tested for the inhibition of Cdc7/Dbf4 at a 25 µM concentration (data not shown). Only one compound, rottlerin, showed significant activity with IC50 of 8.0 µM (Fig. 3). To address the mechanism of the Cdc7 inhibition, the IC50 of rottlerin was determined at ten ATP concentrations in the range 0.1-50 µM. It was found to be essentially unchanged (7.9-8.7 µM). This result indicated a non-ATP competitive mechanism of action. Rottlerin is a natural product that is reported to inhibit a number of different serine/threonine kinases with some specificity to PKC-delta in a predominantly ATP-competitive manner (Gschwendt et al., 1994). The fact that rottlerin behaves as a non-ATP-competitive inhibitor of MCM2 phosphorylation by Cdc7/Dbf4 suggests a different action mechanism. The potency of rottlerin against Cdc7/Dbf4 is relatively low, but its structure can be exploited for designing more potent inhibitors of this important kinase. Due to its selectivity to PKC-delta, rottlerin has been widely used in investigations on the cellular functions of different PKC isozymes at concentrations in the 10-30 µM range (Cross et al., 2000; Chang et al., 2001; Song et al., 2001; Tabakoff et al., 2001). The possibility for inhibiting the cellular activity of Cdc7/Dbf4 under these conditions should be taken into account when interpreting past and future results with rottlerin.

The generic serine/threonine assay platform that is reported here should be widely applicable for the development of high throughput non-radioactive assays to identify kinase inhibitors using protein or peptide substrates. The Eu-labeled antibody that was used in this study was tested for specificity using eight phospho-threonine peptides that contained phosphorylation sites of known serine/threonine kinases (AKT, PKC-epsilon, SOK, p38, MKK7, ERK2, CamKII, CamKIV). They were all specifically recognized by the antibody with a signal to background ratio (measured with phosphorylated and non-phosphorylated peptides) between 24 and 52 (AD0092 product information, PerkinElmer Wallac). We report here an assay that extends the utility of the antibody to protein substrates. This assay platform allows the measurement of kinase activity that phosphorylates multiple sites within a folded protein substrate. Cdc7 is one of these kinases that utilize phosphorylation sites within 9 peptide fragments in vivo (Jiang et al., 1999).

The time-resolved fluorescence format that is reported here can be easily converted to a homogeneous FRET-based assay in order to further reduce sample handling and increase assay throughput. The major advantages of this assay are as follows: (a) The potential applicability to many serine/threonine kinases. (b) The use of commercially available reagents, thus eliminating the need for a generation of substrate-specific antibodies. (c) The possibility to use natural protein substrates as well as peptides.

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References


Cell Signaling Technologies, Catalog No. 9381. Product insert.


