Kinase Assay Based on Thiophosphorylation and Biotinylation

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ABSTRACT

Protein kinases catalyze the transfer of the γ -phosphate group from ATP to a serine, threonine or tyrosine residue of an acceptor protein. These enzymes play an important role in signal transduction. New inhibitors for these enzymes are actively being sought. In this article, we present a novel approach for detecting the activity of protein kinases, which could be useful for the high-throughput screening of chemical libraries. The method is based on the use of ATP γ S instead of ATP in the phosphorylation reaction. This results in the transfer of a thiophosphate group onto a fluorescein-labeled acceptor peptide substrate. The mixture is then treated with a sulfur-reactive iodoacetyl derivative of biotin, which leads to the modification of the nucleophilic sulfur of the thiophosphate group and the generation of a fluorescently labeled, biotinylated molecule. Finally, streptavidin is added to the mixture and it binds to all biotinylated molecules present. The binding of streptavidin to the thiophosphorylated and biotinylated kinase substrate can be conveniently detected by measuring the change in fluorescence polarization of the fluorescent dye attached to the peptide. The detection of kinase inhibitors is demonstrated. The method is completely homogeneous and does not require any separation steps.

INTRODUCTION

Protein tyrosine and serine/threonine kinases are an important class of enzymes involved in the regulation of a number of biological processes. These enzymes are an increasingly significant target for new drug design. Methods for the rapid, high-throughput screening of chemical libraries for the identification of new inhibitory structures against these enzymes are actively being pursued.

Traditionally, the enzyme activity of protein kinases has been assayed by following the transfer of a radioactive phosphate group from [γ - 32 P]ATP to the tyrosine, serine or threonine residue of a suitable protein or peptide substrate (3,15). Following the phosphorylation reaction, the labeled product has to be separated from excess labeled ATP. This approach requires radioactivity, involves multiple steps and is poorly suited for high-throughput screening applications. The scintillation proximity-based approach (1) represents an improvement, but it still has all the disadvantages of radioactive assays.

Several nonradioactive kinase assay approaches have been described that rely on the use of anti-phosphotyrosine antibodies. In one method (2), these antibodies are labeled with an Eu chelate. The substrate is immobilized to the surface of an ELISA plate, and the product of phosphorylation is detected using time-resolved fluorescence following incubation with the labeled antibodies. The main disadvantage of this method is its heterogeneous nature, which does not easily permit the detailed enzymological characterization of the kinase.

Alternatively, a completely homogeneous approach has been described in which the binding of the antibodies to the reaction product is detected by fluorescence polarization (13,14). These methods may not work as well for serine/threonine kinase because of the lack of similar, high-affinity anti-phosphoserine/threonine antibodies.

All kinases can be assayed by separating the substrate from the phosphorylated product, for example by HPLC, capillary electrophoresis, thin-layer chromatography (TLC), ion-exchange chromatography, etc. However, the need for a separation step represents a complication for high-throughput screening applications.

Here, we present an alternative approach for detecting kinase activity that could be useful for the high-throughput screening of chemical libraries. The method does not require the use of radioactivity or antibodies and allows flexibility in the detection scheme. Our feasibility studies were carried out with the cAMP dependent protein kinase A (PKA), but preliminary experiments with other kinases suggest the method will be useful for a wide range of different kinases. Figure 1 shows the general scheme of this new method.

In the first step of the method (see Figure 1), the fluorescein-labeled PKA substrate **1**, known as Kemptide, is phosphorylated using adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) instead of ATP. This results in the generation of the thiophosphorylated product **2**. To the reaction mixture is now added a solution of the iodoacetyl derivative of biotin **3**, and the mixture is allowed to react at room temperature. The sulfur

group of the thiophosphate derivative **2** reacts with the iodoacetyl function of **3** and leads to the formation of the biotinylated, fluoresceinated product **4**. The formation of this product can be assessed in several ways. To develop a completely homogeneous assay, we have chosen to use fluorescence polarization as the analytical tool. Thus, streptavidin was added to the reaction mixture and allowed to bind to all biotinylated species in the reaction mixture, including compound **4**. Binding of streptavidin to **4** results in an increased fluorescence polarization value of product **4** compared to the substrate **1**. The presence of a kinase inhibitor dur-

ing the phosphorylation step is indicated by a final fluorescence polarization value that is lower than that for the control in the absence of an inhibitor.

MATERIALS AND METHODS

Thiophosphorylation of Kemptide

A typical reaction mixture (50 μ L) contained 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 μ M fluorescein-labeled Kemptide (Research Genetics, Huntsville, AL, USA), 500 μ M $ATP\gamma S$ (Sigma, St. Louis, MO, USA) and 1 μ L of the catalytic subunit of cAMP-depen-

Figure 1. General scheme of the kinase assay based on thiophosphorylation and biotinylation.

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dent protein kinase A (Promega, Madison, WI, USA). The approximate final concentration of the enzyme was 700 nM. In inhibition experiments, the PKA inhibitor H-89 (Calbiochem-Novabiochem, San Diego, CA, USA) was added to the reaction mixture at the concentrations indicated below. The mixture was incubated at room temperature for 15 min, and the reaction was terminated by the addition of EDTA to 45 mM. As a negative control, EDTA was added to one of the reaction mixtures before the addition of the kinase. The extent of thiophosphorylation was then analyzed by capillary electrophoresis with fluorescence detection either on a P/ACE System 5500 instrument (Beckman Instruments, Fullerton, CA, USA), using a 50 μ m fused silica capillary with 50 mM borate, pH 8.6, as the separation buffer or on a microfabricated chip as described recently (5). Substrate and product peak areas were integrated, and the extent of substrate phosphorylation was determined from their ratio.

Biotinylation of the Thiophosphorylated Product

To 45 μ L of the thiophosphorylation mixture was added 5 μ L of a freshly prepared 10 mM solution of iodoacetyl-LC-biotin (Pierce Chemical, Rockford, IL, USA) in dimethyl sulfoxide (DMSO). The mixture was left at room temperature overnight and protected from light. The outcome of the biotinylation reaction could be determined at this point by capillary electrophoresis as described above. Note that we used capillary electrophoretic analyses as an analytical tool during the development of the method; they are not part of the final assay.

Fluorescence Polarization Measurements

Polarization measurements were carried out on a FluoroMax-2 (Instruments SA, Edison, NJ, USA) using excitation at 490 nm and emission at 525 nm. An aliquot (1 μ L) of the biotinylation reaction was diluted in 400 μ L of 50 mM HEPES, pH 7.4, and its fluorescence polarization was measured. Four microliters of a 160 μ M solution of

streptavidin were then added to this solution, and the polarization was measured again. In preliminary streptavidin titration experiments, we established that this amount of streptavidin was sufficient to generate a maximal fluorescence polarization signal; the addition of more streptavidin did not increase that signal further.

Calculations

To quantitate the extent of Kemptide phosphorylation from the measured fluorescence polarization values, we used the following equation (9):

$$c_{\text{prod}} = (F_{\text{obs}} - F_{\text{sub}}) / \{(F_{\text{prod}} - F_{\text{obs}})R + F_{\text{obs}} - F_{\text{sub}}\}$$

where c_{prod} is the molar fraction of the thiophosphorylated, biotinylated product; F_{sub} , F_{prod} and F_{obs} are the experimentally measured fluorescence polarization values in the presence of streptavidin for the pure substrate [1], pure product [4] and the mixture thereof, respectively; and R is the ratio of the quantum yields of the fluorescein tag in the two molecules, 4 and 1, in the presence of streptavidin. The values of F_{sub} and F_{prod} were determined from samples that contained pure Kemptide 1 and pure thiophosphorylated, biotinylated Kemptide 4, in the presence of excess streptavidin. The value of R was deter-

mined by measuring the fluorescence polarization of an equimolar mixture of these two compounds (i.e., $c_p = 0.5$) and solving the equation for R .

RESULTS

Figure 2 shows the overlaid electropherograms of a mixture containing approximately 80% of Kemptide 1 and 20% of its thiophosphorylated derivative 2 before and after the treatment with the iodoacetamide derivative of biotin 3. Peak A in both electropherograms is the starting fluorescein-labeled Kemptide, peak B is the thiophosphorylated, biotinylated product 4, and peak C is the product of the PKA-catalyzed reaction, compound 2. As expected, the treatment of the mixture of compounds 1 and 2 with the sulfur-specific reagent 2 led to the quantitative biotinylation of the thiophosphate 2 while compound 1 remained unchanged.

We then studied the effect of streptavidin addition on the fluorescence polarization values of two solutions containing either pure Kemptide 1 or pure biotinylated thiophosphate 4. These two solutions were obtained by PKA-catalyzed thiophosphorylation reactions in the presence or absence of EDTA, followed by treatment with iodoacetyl-LC-biotin. The presence of

Figure 2. Overlaid electropherograms of a mixture of PKA substrate 1 (peak A) and its thiophosphorylated derivative 2 (peak C) before (solid line) and after (dotted line) treatment with the iodoacetyl-LC-biotin 3. Compound 2 is quantitatively converted into compound 4 (peak B), and compound 1 remains unchanged. Separations were carried out on microchips as described (5).

EDTA was expected to completely inhibit the phosphorylation reaction by chelating the Mg^{2+} ; the composition of the two solutions was confirmed by capillary electrophoresis. The fluorescence polarization value for both solutions in the absence of streptavidin was found to be 38–40 mP (P = polarization unit). The addition of increasing con-

centrations of streptavidin resulted in increasing polarization values for the solution of compound **4**, until a maximum signal of 180–185 mP was reached; no change in the fluorescence polarization signal was observed for compound **1**. We found these values to be highly reproducible, but because fluorescence polarization is sensitive to

Figure 3. Correlation between capillary electrophoresis and fluorescence polarization for the analysis of PKA reactions.

Figure 4. PKA inhibition by H-89 as determined by fluorescence polarization measurements in the presence of streptavidin following thiophosphorylation and biotinylation reactions.

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temperature, it is advisable to experimentally measure these two values with every set of kinase reactions.

By mixing aliquots of pure compounds **1** and **2** in different proportions, several solutions were prepared representing kinase reaction products with various degrees of substrate conversion. The exact composition of these mixtures was determined by capillary electrophoresis. The same mixtures were then treated with iodoacetyl-LC-biotin, and finally their composition was analyzed by measurements of their fluorescence polarization signals in the presence of streptavidin. From these values, the exact composition was calculated as described in the Materials and Methods section. Figure 3 demonstrates that the quantitation results from both analytical methods, capillary electrophoresis and fluorescence polarization in the presence of streptavidin, showed excellent agreement.

We then tested the suitability of this approach for the detection of kinase inhibitors by performing PKA reactions in the presence of a range of concentrations (0.5–10 μM) of compound H-89, an ATP-competitive PKA inhibitor. Following a 15 min incubation, the reactions were stopped by the addition of EDTA, treated with iodoacetyl-LC-biotin and analyzed by fluorescence polarization in the presence of streptavidin. Triplicate measurements of each sample were carried out; Figure 4 shows the average polarization values and SD for all samples. From the observed polarization signals the substrate conversion was calculated as described above, and an IC_{50} value of approximately 5 μM was estimated for this inhibitor under the experimental conditions used. Because $\text{IC}_{50} = K_i(1 + [\text{S}]/K_m)$, where $[\text{S}]$ is the ATP γS concentration and K_m is the Michaelis-Menten constant for this cofactor, we

calculated a K_i value of approximately 60 nM (the ATP γS concentration used was 500 μM ; we determined its K_m value to be $\sim 6 \mu\text{M}$ by running PKA assays at different ATP γS concentrations on a microchip as recently described in Reference 5). This K_i value is in very good agreement with the previously reported inhibitory constant of 48 nM for this compound (4).

DISCUSSION

The method presented in this article is based on two previous observations. First, the nucleophilic sulfur of thiophosphates is reactive towards haloacetates. This reactivity has been explored before for labeling of thiophosphorylated peptides (6,10) and nucleic acids (7). Second, a large number of protein kinases (both tyrosine and serine/threonine) accept the nonphysiological sub-

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strate ATP γ S as a replacement of its normal counterpart ATP (6 and references cited therein). We combined these two observations to devise a new, non-radioactive method for the detection of protein kinase activity. We were especially interested in a method that can be carried out in homogeneous phase and could easily be automated for the purposes of high-throughput screening of chemical libraries.

The present method uses fluorescence polarization as the final analytical tool. A somewhat similar protocol using a fluoresceinated, biotinylated protease substrate coupled with a fluorescence polarization detection scheme has recently been reported for measuring protease activity (8). The use of fluorescence polarization is convenient as no separation of substrate and product is required. Detection approaches other than fluorescence polarization can easily be designed. First, the final reaction mixture following the thiophosphorylation and biotinylation can be transferred to a streptavidin-coated mi-

croplate and the biotinylated species (3, 4, and the product of reaction between 3 and ATP γ S) allowed to bind to the solid phase. Following a wash step, the fluorescent signal due to bound 4 can be detected using a fluorescence plate reader. The observed signal will be directly proportional to the concentration of 4, with no interference from the unmodified substrate 1. Second, the starting peptide substrate could be biotinylated and, following the thiophosphorylation, reacted with a haloacetyl derivative of a fluorescent dye. The mixture could then be captured onto a streptavidin-coated plate. Similar approaches using biotinylated peptide substrates of protein kinases have recently been described, but they still require the use of radioactive ATP (12).

An added advantage of this method for kinase assays over those using radioactive ATP is the fact that the concentration of ATP γ S in the assay can easily be varied over a wide range. This can be useful for characterizing the kinetics of kinase inhibitors (i.e., ATP, competitive or not) (11).

The biggest drawback of the present method is the relatively slow rate of the biotinylation reaction, which requires 5–8 h for completion and the use of an excess of the biotinylation reagent. However, the method is completely homogeneous and, if carried out in a microplate format, requires only the addition of certain reagents (iodoacetyl-LC-biotin and streptavidin) to the reaction mixture after the initial set-up. Thus, it represents a viable alternative to existing methods of high-throughput screening of protein kinases.

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