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Short communication

Utilization of luminescent technology to develop a kinase assay: Cdk4 as a model system

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Abstract

Protocols to assess kinase activity generally include radioactive methods, fluorescent polarization technology and the use of specific antibodies. Here, a simple, effective, non radioactive method to measure kinase activity of immunoprecipitated proteins is described. Cdk4, a cell cycle dependent enzyme, was immunoprecipitated from whole cell extracts and used in kinase reactions. This system has been developed taking advantage of the kinase-Glo™ reagent (Promega), based on ATP depletion technology, but with a wider range of applications. The original aim of the commercial kit is the evaluation of kinase activity of highly purified enzymes, while this system enabled the evaluation of native kinases, retrieved by immunoprecipitation. This method was highly homogeneous and did not require any kind of separation or purification as well. Moreover, it was suitable for basic research and may be useful for low-medium throughput pharmaceutical screening of chemical libraries.

Keywords: Kinase assay; Cd4k; Luminescence; ATP depletion

1. Introduction

Assessment of kinase activity is a valuable tool for studying intracellular signaling and evaluating the efficacy of kinase inhibitors employed in cancer treatment. Radioactive methods have been the most used approaches [1–3] so far. On the other hand, several non radioactive methods to evaluate kinase activity have been developed, based on fluorescent polarization technology [4] or on the use of specific anti-phospho-serine, -tyrosine or -threonine antibodies [5]. These techniques show important drawbacks: highly purified enzymes or specific anti-phospho antibodies are necessary, implying high costs and laborious technology, especially when analysing a kinase substrate with multiple phosphorylation sites. Here, an alternative and simple way for determining kinase activity is described, focusing on Cdk4 kinase activity determination in HeLa cells, where the enzyme phosphorylates the retinoblastoma (Rb) protein in G2/M phase [6]. Our system has been developed taking advantage of the kinase-Glo™ reagent (Promega), which evaluates kinase activity of highly purified enzymes. This system has been developed to evaluate native kinases, retrieved by immunoprecipitation, performing the kinase reaction with whole cell extracts immunoprecipitated with anti-cyclin dependent kinase 4 antibody (Ip-Cdk4), Rb protein and ATP in order to obtain phosphorylated Rb protein (pRb). After the reaction, luciferase was added together with its substrate (luciferin), inducing a reaction that produced oxyluciferin and light, by using residual ATP (www.promega.com). Therefore, the depletion of ATP due to the kinase reaction (first step) could be monitored with high sensitivity, since the developed luminescence (second step) could be easily recorded by a luminometer. Moreover, since ATP depletion is a common feature of all

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kinase enzyme reactions, this assay is not restricted to particular kinases, but can be performed to assess kinase activity of virtually any kinase (Ser/Thr and/or tyrosine) and substrate combination.

2. Materials and methods

2.1. Materials

All reagents, if not otherwise specified, were purchased from Sigma–Aldrich (Milano, Italy).

2.2. Cell culture

Cdk4 kinase activity was assessed in the HeLa cell line (doubling time 24 h), purchased from LGC Promochem (Milano, Italy) and maintained in RPMI 1640 supplemented with 10% FBS (EuroClone, Milano, Italy). The cells were serum-starved for 20 h, and then incubated with culture medium supplemented with 10% FBS. Cells were collected in G2/M phase at 20, 22, 24 and 26 h after serum incubation [6].

2.3. Immunoprecipitation and immunoblotting

Cells were resuspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF, 25 μg/ml leupeptin, 10 μg/ml aprotinin, 1% Triton-X 100), and lysed by vortexing for 1 min with an equal volume of glass beads. Samples were then centrifuged for 5 min at 5000 rpm and for 20 min at 13000 rpm. Protein concentration was then determined with BCA Protein Assay Reagent kit (Pierce, Rockford, IL). For immunoprecipitation, 100 μg of protein extracts were incubated with 20 μl protein G sepharose beads (Amersham Biosciences, Milano, Italy) in the presence of 1 μg monoclonal mouse anti-Cdk4 antibody over night at 4 °C in HNT buffer (HEPES 30 mM, NaCl 30 mM, 0.1% Triton-X 100). As control, cell extracts were not added in the mixture (no Ip-Cdk4 control). For immunoblot, Ip-Cdk4 was loaded on a 10% SDS-polyacrylamide gel and transferred on nitrocellulose membranes (Schleicher and Schuell GmbH, Germany). Membranes were blotted with mouse monoclonal anti-Cdk4 and anti-cyclin D1 antibodies (Pharmingen, Milano, Italy). Antibodies bound to membrane immobilized proteins were visualized by SuperSignal West Femto maximum sensitivity substrate (Pierce). All procedures were performed at 4 °C, avoiding sample freezing or boiling. The purity of immunoprecipitated complexes was verified by silver staining (BioRad, Milano Italy).

2.4. Cdk4 kinase assay

Ip-Cdk4 obtained from 100 μg of total protein extract was washed three times in lysis buffer, twice in kinase buffer (50 mM, pH 7.5, 10 mM $MgCl_2$, 250 μM EGTA, 10 mM β-glicerophosphate, 100 μM Na_3VO_4, 1 mM DTT) and resuspended in 30 μl of kinase buffer, optimised to preserve the Cdk4 kinase activity. Kinase reaction was performed adding 20 μl of a mixture containing 0.1 μM ATP (ICN Pharmaceutical, Milano, Italy) and 2 μg Rb peptide (aa773–928; USBiological, Milano, Italy) as substrate in 1× kinase buffer. The reaction was incubated for 30 min at 30 °C and then an equal volume of kinase-GLO™ reagent (Promega, Milano, Italy) was added. As control, kinase reaction was performed with the same samples in the absence of the peptide substrate (no Rb) and with no Ip-Cdk4 control. This is a crucial step since the difference between the luminescence recorded in samples with the substrate and that measured in control samples allows the detection of specific kinase activity, eliminating the background. Samples were then incubated for 10 min at room temperature and the developed luminescence was recorded using the Wallac Victor™ 1420 Multilabel Counter (Perkin-Elmer, Monza, Italy) and expressed as relative light units (RLU).

2.5. Inhibition of Cdk4 kinase activity by indirubin-3′-monoxime

Kinase reactions were performed in cells harvested 22 h after serum release, as described above, in presence or absence of 4 μM of a Cdk4 inhibitor, indirubin-3′-monoxime. As control, kinase reaction was performed with the same samples in the absence of the peptide substrate (no Rb), or in the absence of cell lysate (no Ip-CdK4).

2.6. Statistical analysis

Results were expressed as the mean ± S.D. Student's paired or unpaired *t*-test was used to evaluate individual differences between means. $P < 0.05$ was considered as significant.

3. Results and discussion

The presence of Cdk4 (33 kD) and of its co-factor cyclin D1 (36 kD) in Ip-Cdk4 was demonstrated by SDS-polyacrylamide gel electrophoresis followed by either silver staining (Fig. 1, Panel A) or by immunoblotting for cyclin D1 and Cdk4 (Fig. 1, Panel B). We then studied HeLa cells synchronized using serum-starvation protocol, where Ip-Cdk4 kinase activity was detected at all time points (Fig. 1, Panel C). Approximately 10–15 min after kinase-GLO™ reagent was added, light emission reached maximum intensity and was stable for about 2 h (data not shown). In HeLa cell extracts incubated for 22 h, a 10-fold luminescence reduction was detected in samples containing Rb peptide as compared to samples without substrate (no Rb) ($P < 0.001$) (Fig. 1, Panel C). The same results were obtained by comparing samples containing Rb with no Ip-Cdk4 control sample. There-

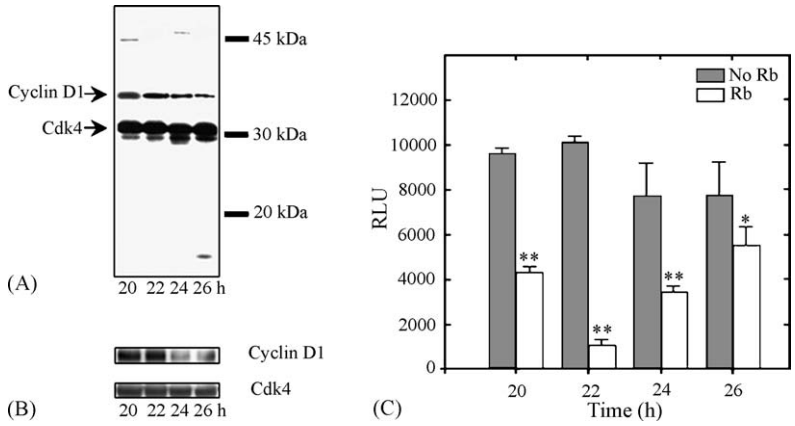

Fig. 1. Analysis of Ip-Cdk4 purity by silver staining (Panel A) and immunoblot (Panel B). (Panel C) Luminescent kinase assay. Ip-Cdk4 was resuspended in 50 μl kinase buffer with 0.1 μM ATP (see Section 2). Reactions contained 2 μg (white bars) or no (grey bars) Rb peptide. Luminescence was recorded on a Wallac Victor 1420 multilabel counter. Values represent the mean ± S.D. of three replicates of three independent experiment. RLU = relative light units; $^{**}P < 0.001$ and $^{*}P < 0.05$.

fore, we choose no Rb samples as control. A five-fold and a four-fold luminescence reduction was detected in samples containing Rb as compared to samples without substrate (no Rb) at 20 and 24 h, respectively ($P < 0.001$) (Fig. 1, Panel C). The extent of luminescence reduction was two-fold at 26 h ($P < 0.05$) (Fig. 1, Panel C). Moreover, no difference was observed among reactions without substrate (no Rb) at all time points (Fig. 1, Panel C). These data were consistent with an increased Cdk4 kinase activity in G2/M phase, in accordance to the results achieved by using a radioactive Cdk4 kinase assay [6], but the use of radioactivity was avoided and background noise was eliminated. Furthermore, the luminescence assay did not require separation of the substrate from the product, as for radioactive methods. We then validated our assay testing an inhibitor of Cdk4 kinase activity, indirubin-3′-monoxime [7]). As shown in Fig. 2, in HeLa cell extracts incubated for 22 h, a 10-fold luminescence reduction was detected in samples containing Rb peptide as compared to samples without substrate (no Rb) ($P < 0.001$), while a 50% reduction was detected in samples containing 4 μM indirubin-3′-monoxime when compared to samples without substrate (no Rb) ($P < 0.001$). These data underline that our assay is highly specific for the immunoprecipitated enzyme (Cdk4), since in the presence of the specific inhibitor, the measured kinase activity is strongly reduced. Moreover, our results are in good agreement with the data of Hoessel et al. [7], who showed an IC50 of 3.33 μM for Cdk4-cyclin D1 inhibition by indirubin-3′-monoxim.

This flexible assay did not require radioactivity or phospho-specific antibodies and is suitable for all immunoprecipitated kinases having a well-characterised substrate in a convenient 96-wells format assay. Moreover, the assay here described could be useful for basic research studies, since the sensitivity of the system allows the detection of small kinase activity due to the low amount of enzyme retrieved by immunoprecipitation, avoiding the need for time-consuming purification processes. The assay specificity was guaranteed by immunoprecipitation with specific antibodies and by substrate choice. The reproducibility of the results depends on the amount of Cdk4 contained in the Ip-Cdk4 complex; therefore, it relies on the efficiency of immunoprecipitation. Consequently, the choice of the antibody is a crucial step for the success of the assay. In particular, the antibody should recognize an epitope not including the catalytic site of the enzyme. However, the reproducibility of this assay has been validated, since three independent experiments showed very similar results in each sample, both before and after ATP depletion due to Rb addition (Fig. 1, Panel C). On the other hand, the

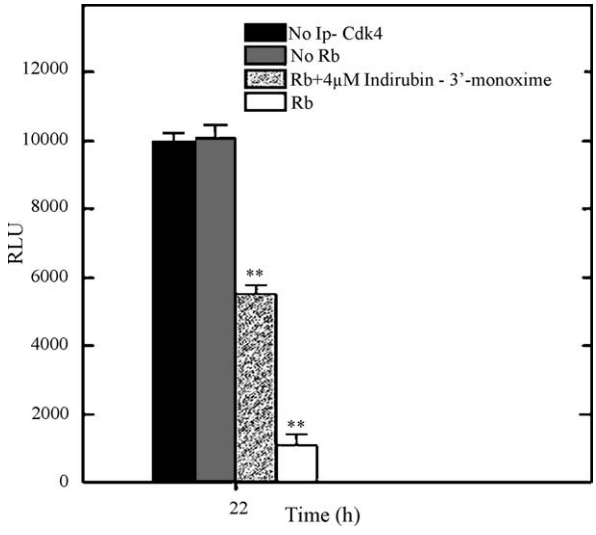

Fig. 2. Inhibition of Cdk4 kinase activity. Ip-Cdk4 was resuspended in 50 μl kinase buffer with 0.1 μM ATP (see Section 2), with 4 μM indirubin-3′-monoxime and 2 μg Rb. As control, kinase reaction was performed with the same samples in the absence of the peptide substrate (no Rb), or in the absence of cell lysate (no Ip-CdK4). Luminescence was recorded on a Wallac Victor 1420 multilabel counter. Values represent the mean ± S.D. of three replicates of three independent experiment. RLU = relative light units; $^{**}P < 0.001$ and $^{*}P < 0.05$.

present method needed an accurate optimisation of reagent concentrations, in particular ATP. In fact, due to the low amount of enzyme retrieved by immunoprecipitation, an optimised quantity of ATP had to be added, in order to induce a measurable kinase reaction. ATP excess might indeed importantly reduce the assay sensitivity since at very high ATP concentrations the low amount of the enzyme utilizes such a small ATP quantity that the RLU difference detected between sample and control is not significant. On the other hand, after optimisation, the assay provided highly homogeneous results. The optimal ATP concentration is that which results in the largest difference in luminescence between the completed kinase reaction wells and wells without the kinase or kinase substrate. Typically, the range of ATP titration is 0.1–1 μM. The sensitivity of this method is highly correlated to the characteristics of the enzyme which has to be immunoprecipitated, and to the substrate specificity. However, in the presented system detection of ATP depletion was no more possible in a 1:2 dilution of Ip-Cdk4 reaction (data not shown), suggesting that the sensitivity of the assay might be improved.

In conclusion, for the first time a valuable and inexpensive method to assess kinase activity of immunoprecipitated proteins based on luminescence technology is presented.

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