KINETIC CHARACTERISATION OF p34^{CDC2}/CYCLIN B KINASE BY SCINTILLATION PROXIMITY ASSAY (SPA).

Program # 2111
Abstract 02904

Spencer - Fry, J.E., Brophy, G., O'Beirne[#], G. and Cook, N.

Amersham Biosciences, Forest Farm, Whitchurch, Cardiff CF4 7YT, U.K. [Telephone: 44 1222 526447, Fax: 44 1222 526230].

Introduction

Cell cycle progression is tightly regulated by a series of protein kinases, phosphatases and proteases[1,2,3]. In particular, p34^{cdc2} kinase is responsible for triggering the G2/M transition[4,5,6]. The Scintillation Proximity Assay (SPA)® format has been developed to accurately measure p34^{cdc2} kinase activity. The assay used a biotinylated peptide derived from the *in vitro* phosphorylation site of the kinase Histone, H1 and used the p34^{cdc2} kinase enzyme from Starfish oocyte (*Marthanterias glacialis*). On phosphorylation of the peptide with [^{33}P]ATP, the reaction products are captured and quantified using streptavidin coated SPA beads. The study examines the kinetic characterisation of the p34^{cdc2}/cyclin B phosphorylation using SPA. Complex analysis of kinetic constants derived from this reaction are compared with those determined from traditional phosphocellulose filter binding assays.

K_m apparent (k_{mapp}) values were determined for several p34^{cdc2} kinase peptide substrates in both their native and biotin labelled forms. This facilitated examination of the effect biotin labelling, essential for capture of the phospholabelled peptide by SPA, on the kinetic parameters determined for that particular substrate. In addition, the progression of the phosphorylation reaction is dependent on the presence of two reaction substrates, ATP and the peptide. The kinetic constants determined for the reaction are hence dependent on the concentration of both ATP and peptide substrates. As a consequence, true k_m (k_m) values were determined for ATP and one of the biotin labelled peptides by assaying over a series of concentrations. Comparison of the kinetic constants derived from SPA and filter binding assays data was made.

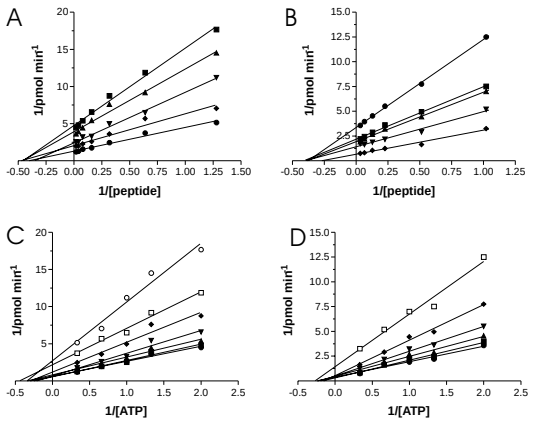

Figure 2. Primary Lineweaver-Burk plots of (A) *filter binding assay data and* (B) *SPA data using ATP concentrations of* (●) *0.5μM,* (■) *0.75μM,* (▲) *1.0μM,* (▼) *1.5μM,* (◆) *3.0μM.*
(C) *filter binding assay data at peptide concentrations of* (●) *50μM,* (■) *25μM,* (▲) *12.5μM,* (▼) *6.25μM,* (◆) *3.13μM,*(□) *1.56μM,*(○) *0.78μM.* (D) *SPA data at peptide concentrations of* (●) *31.25μM,* (■) *15.63μM,* (▲) *7.81μM,* (▼) *3.91μM,* (◆) *1.95μM,*(□) *0.98μM.*

Figure 1

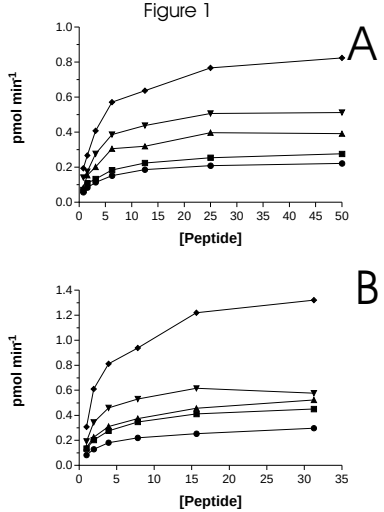

Figure 1. Typical substrate concentration curves obtained and used to derive the kinetic constants with Enzfitter. The rate is determined in picomoles of phosphate transferred per minute versus the peptide concentration. (A) *filter binding assay data and* (B) *SPA data using ATP concentrations of* (●) *0.5μM,* (■) *0.75μM,* (▲) *1.0μM,* (▼) *1.5μM,* (◆) *3.0μM.*

Figure 3

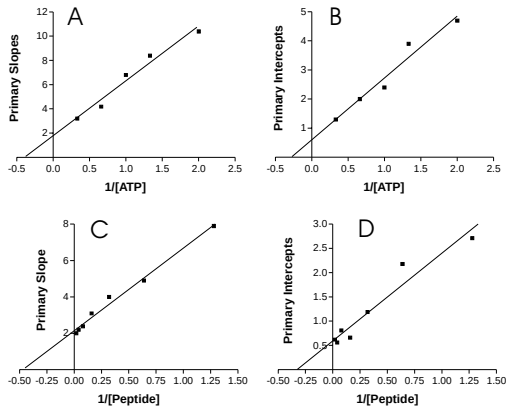

Figure 3. Secondary plots of (A) primary slopes versus 1/ATP, (B) primary intercepts versus 1/ATP, (C) primary slopes versus 1/peptide and (D) primary intercepts versus 1/peptide derived from the primary Lineweaver-Burk plots for the filter binding assay for the determination of Alberty and Dalziel constants.

Table 1.

Peptide	Sequence
HS (9-18)	P-K-T-P-K-K-A-K-K-L
HS (9-18)A14	P-K-T-P-K-A-A-K-K-L
HS (9-18)A16,A17	P-K-T-P-K-K-A-A-A-L

Table 1. Sequences for the Histone H1 derived peptides used as substrates for the kinetic characterisation of p34^{cdc2} kinase.

Table 2.

Peptide	Apparent K_m μM
HS (9-18)	16.2 ± 1.6
BiotinX-HS (9-18)	6.2 ± 1.2
HS (9-18)A16,A17	98.1 ± 10.0
BiotinX- HS (9-18)A16,A17	12.5 ± 2.1
HS (9-18)A14	712 ± 60.3
BiotinX-HS (9-18)A14	68.0 ± 8.5

Table 2. Apparent K_m values determined using the filter binding assay for peptide substrates prior to and following end-labelling with biotin.

Figure 4

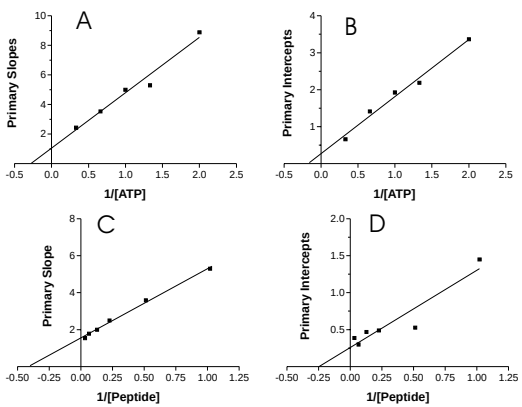

Figure 4. Secondary plots of (A) primary slopes versus 1/ATP, (B) primary intercepts versus 1/ATP, (C) primary slopes versus 1/peptide and (D) primary intercepts versus 1/peptide derived from the primary Lineweaver-Burk plots for the Scintillation Proximity Assay for the determination of Alberty and Dalziel constants.

Table 3.

	True K_m μM	
Substrate	Filter Binding Assay	SPA
Peptide HS(9-18)	3.10	3.90
ATP	3.64	6.00

Table 3. True K_m values determined for the p34^{cdc2} kinase phosphorylation reaction substrates, peptide HS (9-18) and ATP, which were derived from Alberty and Dalziel constants obtained by analysis of the filter binding and SPA data.

Discussion

Sequences of the peptides used in this study are provided in Table 1. Capture and quantification of the phospholabelled peptide by SPA, and several other methods, requires modification of the peptide substrate by the addition of a biotin molecule at the N-terminus. Modification of the substrate in this manner has been shown here to significantly reduce the K_{mapp} value determined for that specific substrate (Table 2.). Thus, an enhancement of the peptide's suitability as a substrate for phosphorylation by p34^{cdc2} kinase is observed.

The K_{mapp} is the kinetic constant most frequently reported in the examination of protein phosphorylation reactions, generally being derived from simple Lineweaver-Burk analysis. However, in a two substrate reaction K_{mapp} may not be the most informative constant, as it will be influenced by the concentration of the second substrate. In this study, true K_m values were determined for ATP and a biotinylated peptide substrate used in the p34^{cdc2} kinase mediated phosphorylation reaction.

The data presented here demonstrates that complex kinetic constants can be obtained by SPA and that these values are comparable to data generated by the standard format filter binding / capture techniques (Table 3).

References

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