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RESEARCH ARTICLE

A FRET-based microplate assay for human protein kinase CK2, a target in neoplastic disease

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Abstract

Besides cardiovascular diseases, cancer represents the major cause of death in developed countries. In many different human tumors, increased activity of serine/threonine protein kinase CK2 has been detected, and recent *in vivo* studies support a direct involvement of CK2 in tumor progression. Therefore, potent compounds to decrease CK2 activity to a non-pathogenic level would be a promising effort toward an antineoplastic therapy. In this study, an alternative to the established radiometric phosphorylation assay for quantification of CK2 activity was developed. For this purpose, the substrate peptide RRRDDDSDDD was coupled at the C-terminus to the fluorophore EDANS (5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid) and at the N-terminus to the quencher DABCYL (4-(4-dimethylaminophenylazo)benzoic acid). This resulted in quenched fluorescence of EDANS due to a FRET-based effect. After proteolytic cleavage of the peptide by elastase, the quenching effect was reduced and, as a consequence, fluorescence was increased. Because elastase is supposed to cleave at the S/D site of the peptide, phosphorylation of serine by CK2 hampered substrate binding of elastase and blocked the increase in fluorescence by proteolytic cleavage. This means that the new assay to quantify human CK2 activity is based on the differential accessibility of the proteolytic cleavage site, which is dependent on kinase phosphorylation. It could be used to measure inhibition of the human target in neoplastic diseases by the compounds TBB (4,5,6,7-tetrabromobenzotriazole) and Emodin.

Keywords: *Protein kinase; phosphorylation assay; CK2; casein kinase 2; FRET peptide; cancer; porcine pancreatic elastase; protease cleavage*

Introduction

Protein kinases play important roles in metabolic and regulatory processes. In general, they catalyze the transfer of the γ-phosphate of adenosine triphosphate (ATP) to specific hydroxyl groups of serine, threonine, or tyrosine residues of peptides or proteins. They are key mediators of eukaryotic signal transduction, controlling the very fundamental cell functions such as differentiation, migration, cell cycle, intercellular communication, and apoptosis, to name only a few. Consequently, dysregulation of kinase activity has vast and severe consequences not only for a single cell but also for the whole individual. The human kinome comprises over 500 kinases, most of which belong to the superfamily of eukaryotic protein kinases (ePKs) and sharing a highly conserved catalytic domain structure[1]. Their catalytic subunits contain a deep hydrophobic ATP binding pocket, enclosed by a bilobal tertiary fold. Most established pharmaceutical approaches to modulate aberrant kinase activity address this binding site by ATP-competitive kinase inhibitors. However, due to the high conservation throughout all ePKs, such kinase inhibitors exhibit a fairly indiscriminate spectrum of specificity[2].

Protein kinase CK2 is a pleiotropic, messenger-independent serine/threonine kinase that is ubiquitously found in a variety of tissues in different eukaryotes. It is a heterotetrameric protein consisting of two alpha and two beta subunits[3]. The active site is located within a basic cluster of multiple lysine residues of the alpha subunit, which is therefore referred to as the catalytic subunit. The beta subunit is found in a single, evolutionary highly conserved isoform, whereas the larger alpha subunit can be identified in three slightly different isoforms, designated CK2α, CK2α′, and CK2α″.

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CK2 was discovered as one of the first protein kinases in the early 1950s[4], but this enzyme is far from being a classic model for protein kinases in general. Some of its exceptional attributes are the constitutive activity, the lack of a regulatory mechanism, the ability to use guanosine triphosphate (GTP) instead of ATP as co-substrate, and the extreme multitude of over 300 CK2 substrates and even more interacting proteins identified to date[5]. Despite its early discovery and its uncovered interactions *in vitro*, the primary physiological role of CK2 and its *in vivo* phosphorylation target(s) remain unclear. Recent reports suggest a prominent role for CK2 in the circadian clock[6,7].

An elevated CK2 level is described as an unfavorable prognostic marker in leukemia[8] and is moreover found in solid cancers of the kidney[9], the lung[10], or the prostate[11], in endometrial carcinomas[12], and in other tumors[13]. A more direct link between CK2 and tumorigenesis was observed in transgenic mouse models. Targeted CK2α overexpression in T cells or cells of the mammary gland resulted in early lymphatic leukemia[14] or transformation of the mammary epithelium, respectively[15]. Bitransgenic mice overexpressing CK2α along with c-Myc even developed lymphoid leukemia *in utero* and died within several days[14]. Moreover, some studies also provided evidence that inhibition of CK2 activity reduces tumor growth and even leads to regression of tumor phenotype in mice[16].

Overall, these and other findings implicate that CK2 is a promising target for therapeutic inhibition in cancer. Besides the possibility to target CK2 expression at a very basal level (e.g. using antisense techniques), the most applicable approach is the exploitation of small molecules to interfere directly with enzyme activity. Despite some years of effort, only a small number of promising CK2 inhibitors are available to date. The most potent compounds are derivatives based on a pyrazolo-triazine scaffold, which have been published recently. The best compound of this series exhibited a K_i (inhibition constant) in the low nanomolar range and an IC_{50} value around 0.1 μM[17]. Besides this scaffold, Emodin, TBB, Fisetin, and IQA are representatives of the main classes of known CK2 inhibitors (Scheme 1). At present, a CK2 inhibitor drug candidate that exhibits anti-tumor activity is entering phase 1 clinical trials (CX-4945; Cylene Pharmaceuticals, San Diego, CA).

Radiometric filtration binding assays represent the standard procedure in kinase inhibitor potency evaluation[18]. In these approaches, the incorporation of a ^{32}P- or ^{33}P-labeled γ-phosphate into a peptide or protein substrate is quantified. The main advantage of this strategy is its universal applicability throughout all kinases. Due to the use of radioisotopes, however, the method requires special handling, disposal, and spatial needs, as well as a series of post-reaction processes, making it laborious and not easy to use in high throughput screening (HTS). Moreover, in most cases, it is difficult to determine the K_m (Michaelis constant) by the radioactive isotope approach, which is indispensable for K_i value calculation. Therefore, alternative approaches to determine kinase activity could be a solution, and the aim of the current study was the development of a CK2 activity test based on fluorescence (or Förster) resonance energy transfer (FRET) to circumvent the restrictions of radiometric assay methods.

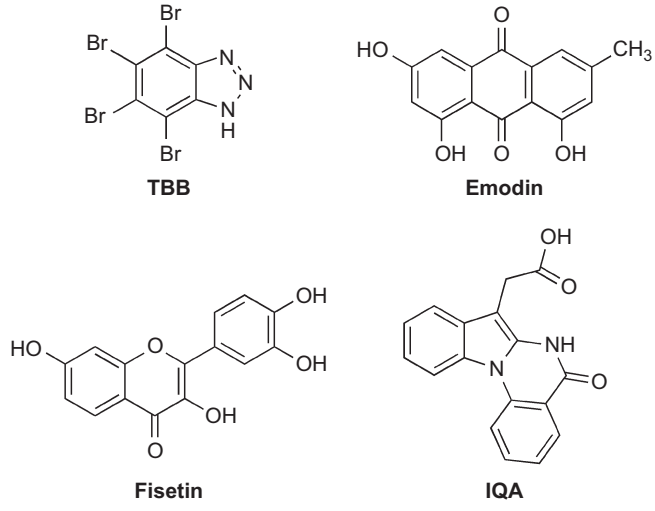
FRET describes the process by which a fluorophore in electronic excitation (the donor) transfers its energy to a second molecule in close proximity (the acceptor) by long-range dipole–dipole interaction[19]. The acceptor can be a fluorophore, but it is not mandatory. It can instead be a quencher whose absorption spectrum largely overlaps with the emission spectrum of the donor. FRET donor/acceptor pairs attached to peptides or proteins are often used in studies of protease characterization (e.g. reference 20). This kind of study deploys FRET to detect the physical separation of fluorescence donor and acceptor to draw a conclusion about protease activity. FRET facilitates monitoring of spatial drawing together or detachment of a FRET pair or two molecules, which are labeled with a FRET donor and acceptor. In this context, the FRET effect can be regarded as a "molecular tape measure."

Because human protein kinase CK2 phosphorylates a single serine residue within a given substrate peptide, and elastase is supposed to cleave the peptide at this site[21,22], which should be hindered by phosphorylation, this was used to set up a FRET-based kinase assay.

Materials and methods

Reagents

Porcine pancreatic elastase was purchased from Lee Biosolutions (St. Louis, MO). The substrate peptide for human CK2 with the amino acid sequence RRRDDDSDDD, which is commonly used for activity measurements[23], was synthesized by the Biologisch-Medizinische Forschungszentrum (BMFZ) of the Heinrich-Heine University, Düsseldorf, Germany. It was modified to yield two forms. Either it was solely labeled with EDANS (5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid) at the C-terminus (referred to as E-peptide) or it was labeled with



Scheme 1. A selection of potent CK2 inhibitors.

EDANS (C-terminus) in combination with DABCYL (4-(4-dimethylaminophenylazo)benzoic acid) at the N-terminus (referred to as D/E-peptide)[24]. Both peptides were purified by high performance liquid chromatography (HPLC).

Preparation of recombinant human protein kinase CK2

Protein kinase CK2 holoenzyme was purified according to a protocol by Grankowski *et al.*[25] with modifications. CK2α- (CSNK2A1) and β- (CSNK2B) subunits were expressed in *Escherichia coli*[26]. Open reading frames of the corresponding genes were expressed in a pT7-7 plasmid system using *E. coli* BL21(DE3) as the host strain for each subunit in a separate approach. Freshly transformed bacterial cells were grown overnight in lysogeny broth (LB) medium at 37°C to the stationary phase. For each CK2 subunit strain, 6 L of LB medium were inoculated and cultivated at 37°C until an OD_{500} (optical density at 500 nm) of 0.6 was reached. Protein expression was induced by the addition of 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside; final concentration) and incubated for 6 h at 30°C (α-subunit) or 3 h at 37°C (β-subunit). Bacterial cells were harvested by centrifugation (6000 × *g*, 4°C, 10 min), disrupted by sonification (3 × 30 s on ice), and cell debris was removed by centrifugation. The crude bacterial cell extracts of the two expression strains were merged and subjected to a three-step column purification procedure as described earlier[26]. Fractions were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and CK2 activity was determined by the radiometric assay as described below. Fractions containing CK2 activity were pooled and stored in aliquots at –80°C. By this procedure, in total, an amount of 50 mg CK2 holoenzyme with a protein concentration of 1 mg/mL was obtained.

Radiometric ^{32}P-based phosphorylation assay

CK2 holoenzyme (2.5 U) was mixed with kinase buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)) in a total volume of 20 µL. Reaction was started by adding 30 µL assay buffer (25 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP, 0.19 mM substrate, and 0.6 µCi [γ-^{32}P] ATP). Due to the three arginine residues at the N-terminus of the substrate peptide, it can be purified by ion exchange paper chromatography. After an incubation of 15 min at 37°C, the complete reaction mixture was spotted onto a P81 phosphocellulose ion exchange paper (Whatman, Maidstone, UK), washed three times with excess phosphate (85 mM H_3PO_4), and a final time with pure ethanol. The filter was dried and the associated radioactivity was determined by a Tricarb scintillation analyzer (Packard/PerkinElmer, Waltham, MA).

FRET-based phosphorylation assay in 384-well format

CK2 holoenzyme (50 U) was mixed with kinase buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) in a total volume of 4 µL in black 384-well plates (Nunc, Langenselbold, Germany). In the case of inhibitor testing, the compound was initially dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mM and then mixed with kinase buffer to reach a final concentration of 10 µM in the kinase assay. A 50 U CK2 enzyme solution was pre-incubated with 4 µL kinase buffer/inhibitor-mix for 10 min at 37°C. Reaction was started by the addition of 6 µL assay buffer (25 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP, 0.19 mM D/E-peptide (EDANS- and DABCYL conjugated peptide RRRDDDSDDD)). After incubation for 60 min at 37°C in a humidified atmosphere, 10 mU porcine pancreatic elastase was added to yield a final volume of 40 µL (1 mM Tris/HCl, pH 8.5) per well. Fluorescence values were traced for 30 min by a Mithras LB 940 microplate reader (Berthold, Germany) with an excitation filter of 355 nm (bandwidth: 40 nm) and an emission filter of 535 nm (bandwidth: 25 nm). Values are reported as mean ± SD ($n = 3$). Statistical analysis was conducted using an unpaired Student's *t*-test. A value $p < 0.05$ was considered to indicate a statistically significant difference in fluorescence intensity. Data were calculated with Microsoft Excel 2004.

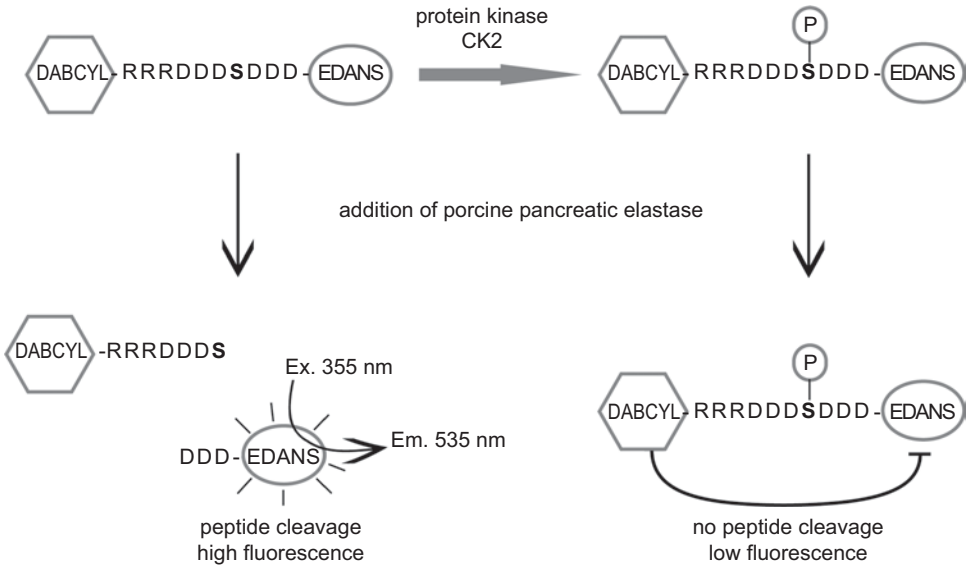
Fluororescence spectra

Fluorescence spectra were recorded using an LS-5B luminescence spectrometer (PerkinElmer, Waltham, MA) and CFS control software with an EDANS-peptide concentration of 10 µM.

Results and discussion

Key concept

The new concept for determination of human CK2 activity is based on a differential protease cleavage rate of phosphorylated vs. non-phosphorylated forms of a kinase substrate peptide (Scheme 2). A similar approach using a tyrosine kinase and chymotrypsin has been described before[27]. The common CK2 substrate peptide RRRDDDSDDD[23] was labeled with a fluorophore (EDANS) at the C-terminus and a quencher (DABCYL) at the N-terminus ("D/E-peptide"). In this peptide, the EDANS-fluorescence signal is almost completely quenched by DABCYL. Within the peptide, a cleavage site for porcine pancreatic elastase can be found that is in general characterized by an uncharged amino acid followed by a charged amino acid[21,22]. In the CK2 substrate peptide, this is represented by the amino acid motif S/D. In case the serine is phosphorylated by CK2, it will be negatively charged, and as a consequence, the peptide will no longer be cleaved by elastase. This means, in summary, that high activity of CK2 will lead to a maintenance of the FRET effect and a reduced fluorescence signal, and low CK2 activity will lead to higher elastase cleavage of the peptide and hence to higher fluorescence values. In other words, in the non-phosphorylated state, the S/D site is recognized by pancreatic elastase and the Ser-Asp peptide bond is cleaved. This event spatially separates the quencher, DABCYL, from the fluorescence donor, EDANS,



Scheme 2. Concept of the CK2 FRET phosphorylation assay. The test is based on a different accessibility of a protease cleavage site within a CK2 peptide substrate depending on its phosphoryation status (P, phosphorylated). The peptide is labeled at both ends, with a fluorescence donor (EDANS) at the N-terminus and fluorescence acceptor (DABCYL) at the C-terminus. Upon cleavage of the non-phosphorylated peptide by pancreatic elastase, the fluorophore and the quencher are spatially separated, resulting in recovery of the donor's fluorescence. The protease recognition site within the phosphopeptide is poorly recognized by pancreatic elastase, leading to a lower enzymatic hydrolysis rate and consequently to a reduced fluorescence intensity.

which results in an increased fluorescent yield at 535 nm after excitation at 355 nm. In the phosphorylated state, the protease recognition site is altered, the cleavage efficiency is lowered, and the EDANS fluorescence is quenched by DABCYL. Although in the present study the molecular basis of the differential protease sensitivity was not further analyzed, it can be assumed that either steric or electrostatic parameters might be involved in the reduction of protease activity[27].

Spectral properties of the EDANS-coupled peptide and validation of FRET peptide phosphorylation by CK2

To combine the fluorophore EDANS with the quencher DABCYL is a commonly used strategy in FRET studies. To find out the correct fluorimeter setup, and to exclude any effect of the amino acid sequence of the CK2 substrate on the fluorescence of EDANS, an excitation- and an emission-spectrum of the EDANS-coupled peptide RRRDDDSDDD–EDANS was recorded (Figure 1). The observed excitation maximum (341 nm) as well as the observed emission maximum (494 nm) was in absolute agreement with the spectroscopic characteristics of the free EDANS molecule, as described before[24].

In the next step, we tried to find out whether modification of the substrate peptide RRRDDDSDDD by DABCYL and EDANS has any influence on the phosphorylation efficiency by human CK2. For this purpose, the D/E-peptide was used as a substrate in radiometric phosphorylation experiments with the recombinant CK2 enzyme. As a control, the unmodified peptide RRRDDDSDDD was used as a substrate as well. As can be seen in Figure 2, there was no difference in substrate associated [γ-^{32}P] activity between the D/E-peptide and the unmodified peptide, which means that protein kinase CK2 accepts both substrates without restrictions.

Elastase cleavage of the D/E-peptide

The crucial step in the presented concept for CK2 activity determination is cleavage of the D/E-peptide by porcine pancreatic elastase. An optimal protease concentration ideally gives rise to a strong signal, while maintaining the highest difference possible between the phosphopeptide and the non-phosphorylated peptide. To optimize the protease concentration, the non-phosphorylated D/E-peptide was incubated with a series of different elastase concentrations and the increase in fluorescence due to proteolytic separation of fluorophore and quencher was recorded by endpoint measurements after 30 min of incubation. As shown in Figure 3, fluorescence significantly increased starting with a concentration of 0.1 mU elastase per 50 µL reaction volume per well of a 384-well plate. A plateau was reached with approximately 1 mU elastase per well, and a further increase of fluorescence was not possible to obtain, even when a concentration of 100 mU elastase was applied. This means that a stable saturation of fluorescence development due to the loss of FRET was reached with 1 mU per well and, to be on the safe side, an elastase activity of 10 mU per well was chosen for further experiments.

Phosphorylation of the D/E-peptide results in reduced elastase cleavage

To find out whether phosphorylation of the D/E-peptide by CK2 leads to reduced cleavage by porcine elastase, the peptide was incubated with the recombinant kinase before it was subjected to proteolysis. The conditions for

phosphorylation of the D/E-peptide by CK2 were identical to those described above for the standard radiometric assay, but the reaction volume was reduced to 10 µL. To exclude any potential unspecific effects of components of the kinase assay mixture or the kinase itself on elastase activity, an identical experiment was performed without the addition of ATP. This should result in a non-phosphorylated peptide, which could be cleaved without restriction by porcine pancreatic elastase. The resulting fluorescence values of the two samples after incubation with elastase are given in Figure 4. Phosphorylation of the D/E-peptide (+ ATP) clearly promoted maintenance of the FRET effect and resulted in a reduced fluorescence intensity of 51,820. In the non-phosphorylated peptide sample (– ATP), a significantly higher fluorescence (60,180) was detected ($p < 0.05$). This substantial difference in fluorescence can only be due to phosphorylation of the serine residue in the D/E-peptide as a consequence of CK2 phosphotransferase activity. In conclusion, CK2 activity could be monitored *in vitro* using the described FRET-based method.

A second aim of this study was to estimate the potential of human CK2-inhibiting compounds. Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) and TBB (4,5,6,7-tetrabromobenzotriazole) are known CK2 inhibitors with IC_{50} values around 0.8 µM and 0.5 µM, respectively[28]. Evaluation of these two inhibitors in the FRET-based kinase assay established here should provide insights into whether the test is appropriate for inhibitor testing. Final concentrations of 10 µM, commonly used in initial CK2 inhibitor assessments, resulted in an effective increase of fluorescence up to the level of the non-phosphorylated peptide (Figure 4). Emodin showed a fluorescence of 60,129 and TBB a value of 58,886. By inhibition of CK2, the two compounds avoid phosphorylation of the D/E-peptide and lead to a higher cleavage rate by porcine pancreatic elastase and, finally, to a higher fluorescence level when compared to the D/E-

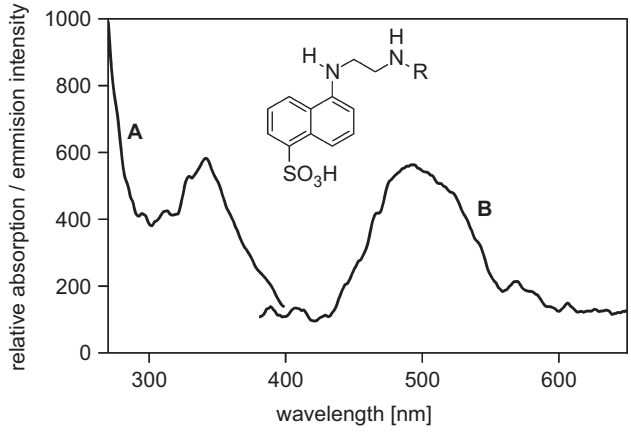


Figure 1. Fluorescence excitation/emission spectrum of the EDANS-coupled peptide. Spectroscopic parameters of the EDANS-coupled peptide (molecular structure depicted; R = RRRDDDSDDD) were derived from its fluorescence spectrum. The excitation spectrum (A) was recorded using an emission wavelength of 487 nm and had a maximum at 341 nm. The emission spectrum (B) was recorded using an excitation wavelength of 342 nm and showed a broad peak with a maximum at 494 nm.

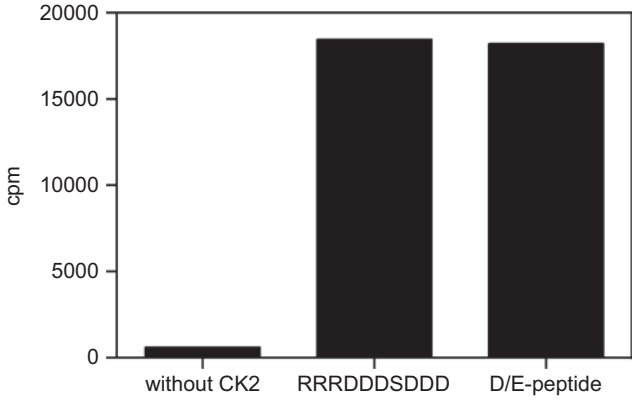


Figure 2. Radiometric CK2 activity assay. The DABCYL- and EDANS-labeled peptide (D/E-peptide) was subjected to a radiometric phosphotransferase assay. Comparison with the unlabeled peptide showed qualification of the D/E-peptide as a CK2 substrate.

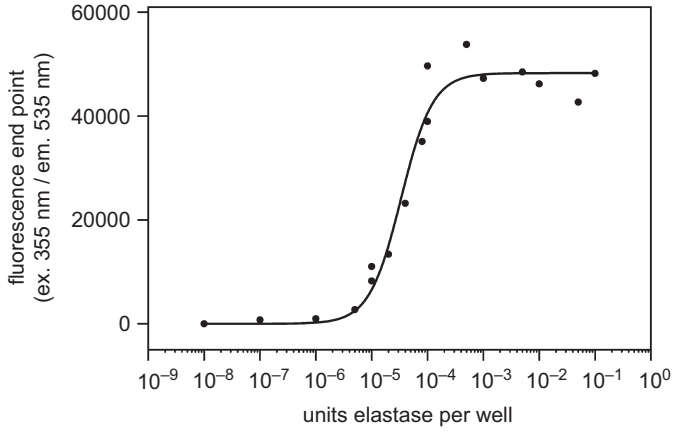


Figure 3. Kinetic analysis of pancreatic elastase parameters. Different units of porcine pancreatic elastase were incubated with D/E-peptide. The developing loss of FRET was measured as the fluorescence intensity (ex. 355/em. 535 nm) after 50 min of incubation.

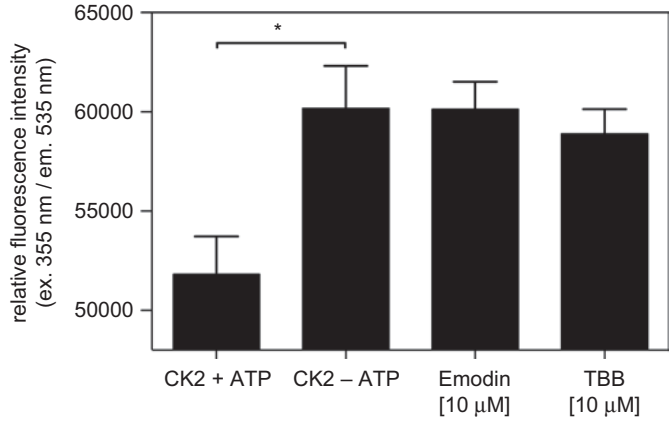


Figure 4. FRET-peptide based CK2 activity determination. CK2 phosphorylation of the D/E-peptide (+ ATP) significantly reduced the resulting fluorescence after elastase incubation ($p < 0.05$). Interference of CK2 activity by the well-known inhibitors Emodin and TBB suppressed CK2 phosphorylation. In this case, a higher fluorescence, which was on the same level as the sample with inactive CK2 (– ATP), could be observed.

phosphopeptide. It was at the same level as found for the sample with CK2, but without ATP.

In summary, these findings confirm that the described FRET-based assay enables analysis of CK2 phosphotransferase activity and the inhibitory potential of a compound as well. In order to obtain quantitative activity data, a further assay improvement is required, which could facilitate IC_{50} and K_i value determinations. Due to the 384-well format it is ready to be applied in high-throughput CK2 inhibitor screening. By this miniaturized design, the setup is not only rapid and safer to use, when compared to a radiometric test, but it is also cheaper in consequence of the substantially reduced kinase reaction volumes. It can provide a complementary tool to further strengthen efforts toward the development and evaluation of novel CK2 inhibitors as potential anti-cancer therapeutic agents.

Declaration of interest

The authors declare no conflict of interest.

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