To make a short story long: recombinant expression and HTS assay development for complex multi-domain protein kinases

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Introduction

Lead discovery programmes for protein kinases often rely on recombinant versions encompassing solely their catalytic domain. However, kinases are complex multi-domain enzymes whose catalytic activity is largely modulated by their ancillary regions. Therefore, we have applied our expertise in recombinant protein production in support to lead discovery programmes on two multifunctional protein kinases:

- Citron Rho-Interacting Kinase (CIT), an AGC kinase structurally homologous to ROCK, which plays a key role in cytokinesis during mitosis.

CIT is a 2027-aa protein kinase, which is conventionally used in compound testing as 449-aa short kinase domain lacking the accessory regions;

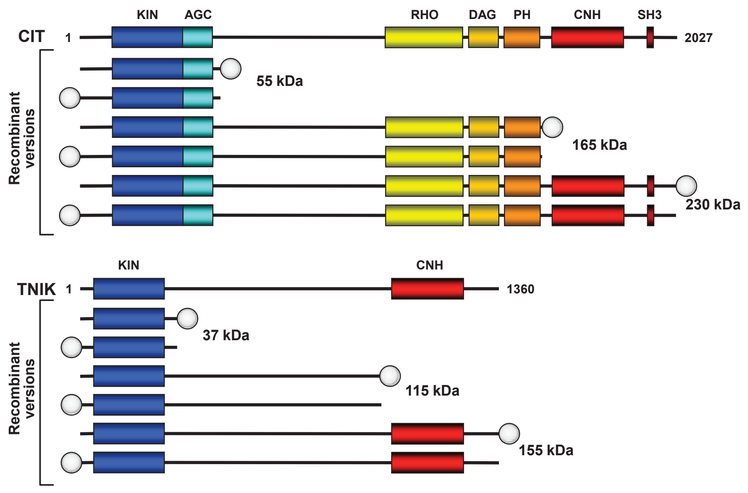
- TRAF2 and NCK-Interacting protein Kinase (TNIK), a GCK family member essential for activation of WNT signalling pathway.

TNIK is a 1360-aa protein generally obtained as 367-aa short version, which encompasses exclusively the catalytic domain.

Conclusions

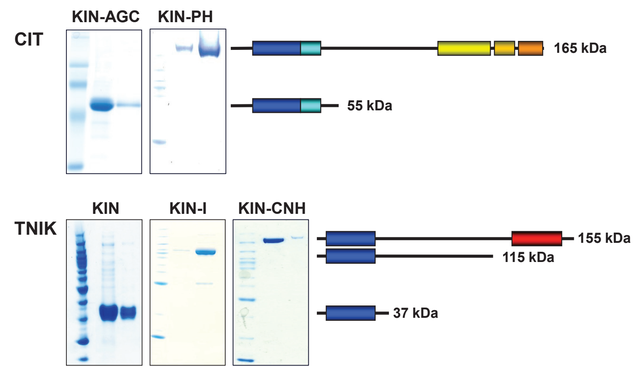
- Permissive conditions for protein production and enzymatic activity of extended versions of CIT and TNIK were identified: CIT was successfully expressed as truncated version of 165 kDa, encompassing over 75% of its entire primary structure, while TNIK was expressed as 155 kDa full-length enzyme;
- Catalytic activity of CIT and TNIK was configured with homogeneous luminescence and fluorescence-based assays in 384 well/plate format suitable for HTS;
- Effective peptide substrates were identified by screening a library of surrogate substrates and the kinetic properties of the enzymatic reactions were fully characterized;
- Expression and purification of CIT and TNIK were up-scaled to achieve a proof-of-principle of potential HTS campaigns on a collection of over 2,000,000 compounds, leading in both cases to homogeneous batches of active kinases;
- This study supported the possibility to overcome the major bottlenecks in the production of long kinase recombinant forms, to ensure that drug discovery programmes are performed with proteins more closely preserving the structural and functional properties of the native enzymes. Moreover, the interplay of the accessory domains with the active site is expected not only to influence the sensitivity to orthosteric molecules, but also to provide potential allosteric sites to target the kinase activity through novel and underexplored mechanisms.

1 CIT and TNIK: protein structures and recombinant versions



2 Recombinant expression and purification

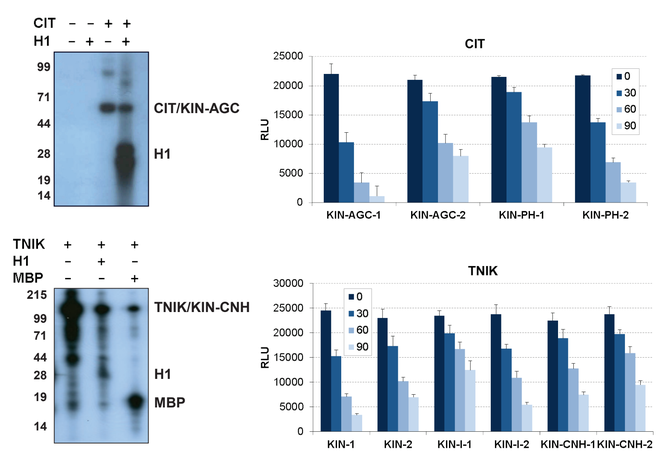
- Recombinant proteins were expressed in insect cells with the baculovirus system
- The most effective expression condition for each chimeric version was identified by screening twelve alternative combinations
- Purification was performed by tag affinity chromatography



- All recombinant versions were purified to near homogeneity, apart from full-length CIT versions, which were recovered exclusively in the insoluble fraction of the cell lysate and thus deprioritized
- The position of the tag had a remarkable effect on the production yield
- Kinase-domain versions were produced at a 3-10 times higher yield compared to longer versions

3 Proof-of-activity

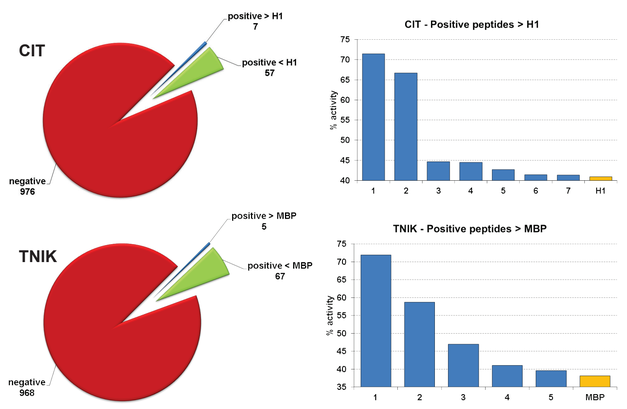
- CIT and TNIK activity were first assayed with radioactive kinase assay using generic substrates
- Activity was than assessed with a luminescence-based coupled system in 384 well/plate format



- CIT and TNIK underwent autophosphorylation and efficiently phosphorylated H1 and MBP, respectively
- Based on expression yield and activity data, CIT-KIN-2 and TNIK-CNH-1 were prioritized for HTS assay development

4 Identification of an effective substrate

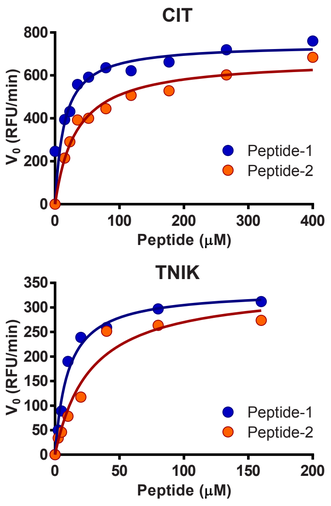
- Prioritized CIT and TNIK recombinant versions were used to screen a library of 1040 peptides enriched in S/T/Y (11-aa)
- H1 and MBP were included as reference substrates for CIT and TNIK reactions, respectively



- Screening of the peptide library identified 6-7% peptides as putative substrates for CIT and TNIK
- Seven peptides for CIT and five peptides for TNIK were identified as more effective substrates than H1 and MBP, respectively

5 Kinetic parameters

- Kinetic parameters for the most effective peptide substrates were determined using a fluorescence-based assay in 384 well/plate format



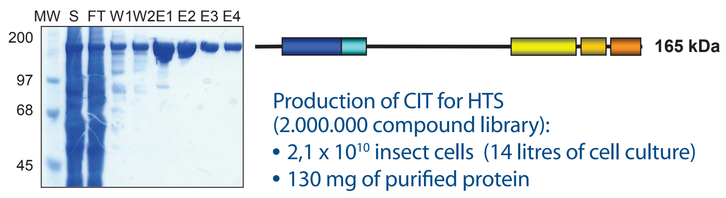
CIT	K_m [μM]	k_{cat} [sec^{-1}]	k_{cat}/K_m [$\mu M^{-1}\ sec^{-1}$]
Peptide-1	14,4	0,63	0,044
Peptide-2	33,1	0,38	0,011

TNIK	K_m [μM]	k_{cat} [sec^{-1}]	k_{cat}/K_m [$\mu M^{-1}\ sec^{-1}$]
Peptide-1	10,1	0,46	0,045
Peptide-2	27,2	0,32	0,012

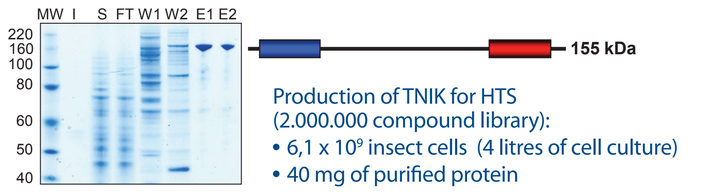
- One peptide substrate was prioritized for CIT and TNIK according to the kinetic parameters, which resulted 3-4 times more effective than generic substrates H1 and MBP, respectively

6 Production of CIT and TNIK long-forms for HTS

- Expression and purification of CIT and TNIK were up-scaled to achieve a proof-of-principle of potential HTS campaigns on a collection of over 2,000,000 compounds



Production of CIT for HTS (2.000.000 compound library):
- 2,1 x 10^{10} insect cells (14 litres of cell culture)
- 130 mg of purified protein



Production of TNIK for HTS (2.000.000 compound library):
- 6,1 x 10^{9} insect cells (4 litres of cell culture)
- 40 mg of purified protein

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