Dimerisation is essential for Kinase Activity

A new and simple Method for Tyrosine Kinase Characterisation

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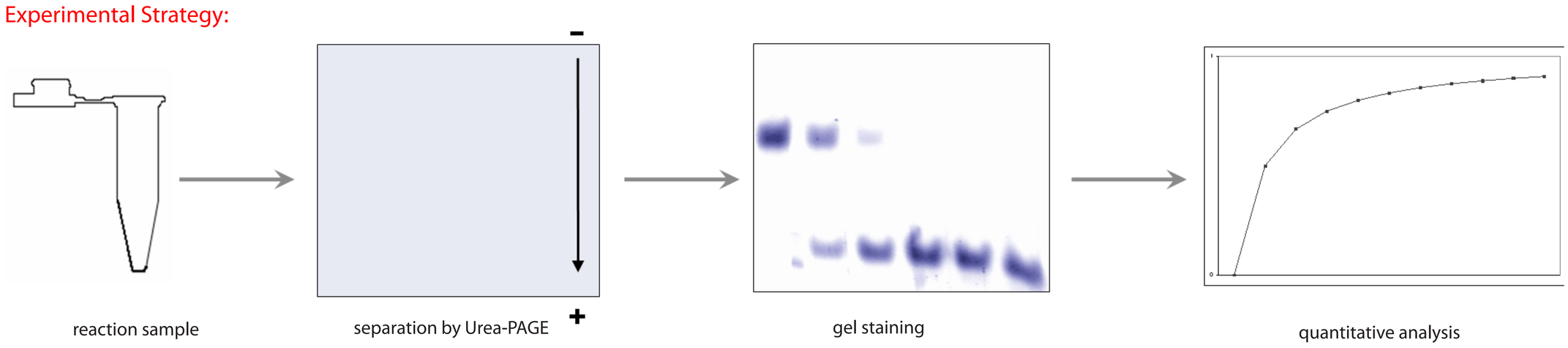
Abstract:

It is known that receptor tyrosine kinases (RTKs) are activated by ligand-induced dimerisation and subsequent autophosphorylation[1]. Consequently, a good indicator of kinase activity is to ascertain the kinetic parameters of substrate phosphorylation reactions. This has generally been done by determining the P^{32} incorporation, immunoblotting with phosphotyrosine-specific antibodies or phosphorylation of the synthetic peptide $(E_4Y)_n$. These methods are rather inconvenient, inaccurate or time-consuming.

Thus we present a fast, new method to monitor the activity of RTKs. We have developed an ideal substrate that is phosphorylated on exactly one tyrosine residue by all tested RTKs. Furthermore, our method of analysis exploits the observation that tyrosine phosphorylation of the peptide substrate (PS^{RTK-1}) causes a mobility shift in Urea-PAGE. Significantly, phosphorylation of PS^{RTK-1} by serine/threonine kinases was not observed. Quantification of the phosphorylated substrate was performed by optical analysis of the amount of dyed protein on the gel.

The presented method is even sensitive enough to allow kinetic studies, particularly, analyse the initial rates of the monomeric and dimeric TrkB receptor kinases. Our results show at least a 5-fold increase of the initial rate by dimerisation of TrkB.

Experimental Strategy:



I. PS^{RTK-1} as an ideal substrate for kinetic studies on RTKs

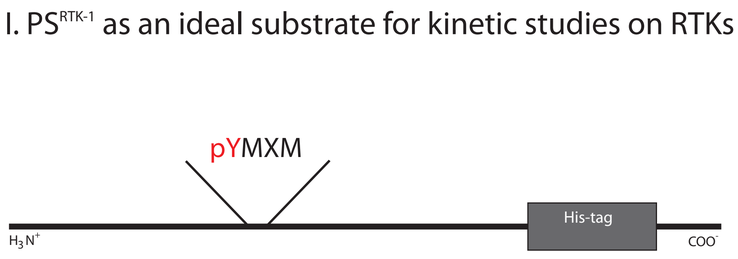


Fig.1a: The peptide substrate 1 for RTKs (PS^{RTK-1}) has 85 amino acids and a molecular weight of 9.2 kDa. It contains the single phosphorylation motif YMXM and possesses a carboxyterminal hexa-histidine tag.

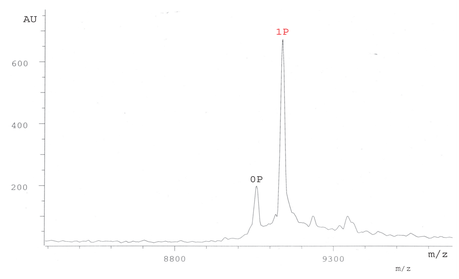


Fig.1b: MALDI-TOF analysis after phosphorylation of PS^{RTK-1} with the IGF receptor kinase for 60min. 10% of the peptide remains unphosphorylated (0P). 90% of the peptide have incorporated exactly one phosphate (1P).

- PS^{RTK-1} is a highly pure recombinant peptide.
- Exactly one Tyr-phosphorylation site per molecule enables precise quantification of phosphate incorporation.

II. Phosphorylated and unphosphorylated PS^{RTK-1} can effectively be separated by electrophoresis

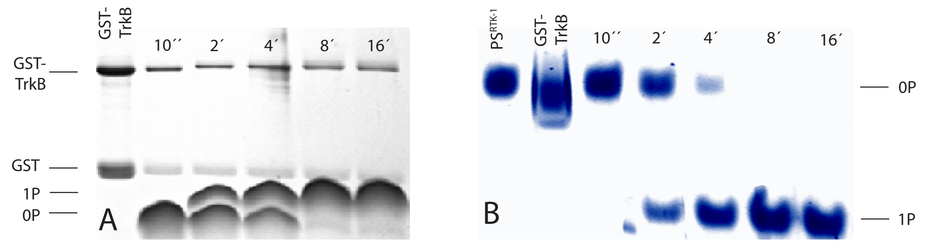


Fig.2: Time-dependence of PS^{RTK-1} phosphorylation with 200 nM GST-TrkB and 50 µM of PS^{RTK-1}. Reactions were stopped in the appropriate sample buffer at the times indicated and separated by PAGE. Identical reactions were analysed by 12% SDS-PAGE (panel A) and 7.6% Urea-PAGE (panel B). Gels were stained with Coomassie Brilliant Blue G. Phosphorylated (1P) and unphosphorylated (0P) PS^{RTK-1} were separated by SDS-PAGE as well as Urea-PAGE. Urea PAGE yielded a better separation that enabled subsequent quantification.

- Urea-PAGE yields an excellent separation of phosphorylated and unphosphorylated PS^{RTK-1} and thereby enables quantification.

Kinetic Studies on the TrkB Receptor Kinase

III. Analysis with PS^{RTK-1} reveals: Dimerisation is essential for the activation of TrkB

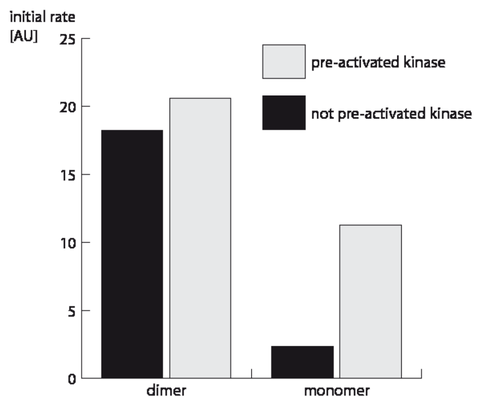


Fig.3: Phosphate incorporation into PS^{RTK-1} by monomeric and dimeric TrkB, analysed by Urea-PAGE. Pre-activated TrkB was autophosphorylated prior to the assay which contained 200 nM TrkB and 100 µM of PS^{RTK-1} as a substrate. The initial rate of the dimeric TrkB was 6.3-fold greater than that of the monomeric kinase. Monomeric TrkB that has previously been activated by autophosphorylation shows a higher initial rate, indicating that dimerisation is essential for kinase activation by autophosphorylation. Our dimerisation model exploits artificial dimerisation via a GST-tag and uses $(His)_6$TrkB as a model for a monomeric kinase.

IV. PS^{RTK-1} is specifically phosphorylated by tyrosine kinases

Kinase	PS^{RTK-1} phosphorylation	K_M for peptide substrate	peptide substrate
TrkB	✓	100µM	PS^{RTK-1}
IGF1 kinase	✓	249µM 79µM 50µM 178µM	RKGNGDG**YMPM**SPKSV KKRVDPNG**YMMM**SPSGV KKKLPATGD**YMNM**SPVGD KKSRGD**YMTM**QIG
EGFR	✓	150µM 440µM 800µM	RRLEEEEEA**Y**G LIEDAE**Y**TA DRV**Y**IHPF
Akt/PKB	✗	n.d.	IRS1-fragment

Table1: PS^{RTK-1} is a substrate for all tested RTKs. No phosphorylation by Ser/Thr kinases, such as Akt, was observed. TrkB has a K_M of 100 µM for PS^{RTK-1}, which was determined by the presented method. The K_M is within the same order of magnitude in comparison to other known peptide substrates[2,3].

V. Quantitative analysis of the stained gel provides kinetic data

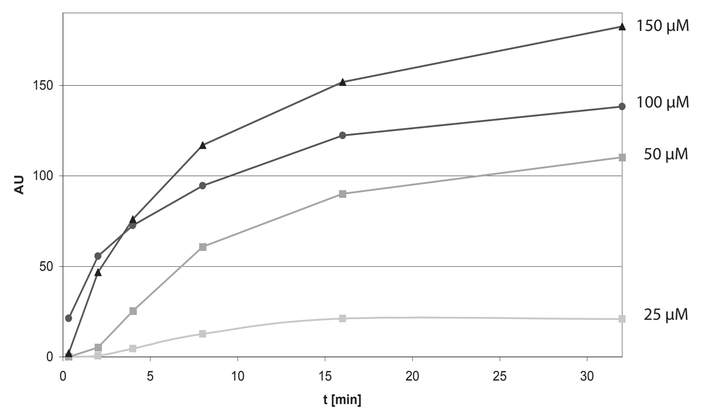


Fig.5: Time-course of phosphorylation of PS^{RTK-1} using 200nM of GST-TrkB and the indicated concentrations of PS^{RTK-1} (25-150 µM). Samples were taken at 15´´, 2´, 4´, 8´, 16´ and 32´. Kinetics were analysed by determination of the stained bands of phosphorylated PS^{RTK-1} after Urea-PAGE.

Summary:

- We obtain a highly pure physiological peptide substrate.
- Usually only serine phosphorylation shows a mobility shift. However, using this method we also observe a mobility shift for tyrosine phosphorylation.
- Neither radioactive labelling nor immunoblottig are required to detect phosphorylation.
- Kinetics of substrate phosphorylation reactions can easily be assessed by phosphorylation of the peptide substrate for RTKs, named PS^{RTK-1}.
- We propose PS^{RTK-1} as an ideal tool to screen solutions and lysates for active tyrosine kinases.
- Dimerisation of the TrkB receptor kinase is essential for activation by autophosphorylation.

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References:
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(2) Hunter, T. and M. S. Sefton, Eds. (1991). Methods in Enzymology. Protein Phosphorylation. San Diego, Academic Press, Inc.
(3) Xu, B., V. G. Bird, W.T. Miller. (1995). J Biol Chem 270(50): 29825-30.