Available online at www.sciencedirect.com
SCIENCE DIRECT®
Methods 35 (2005) 44–53
METHODS
www.elsevier.com/locate/ymeth

Substrate-trapping techniques in the identification of cellular PTP targets

C. Blanchetot*, M. Chagnon, N. Dubé, M. Hallé, M.L. Tremblay

McGill Cancer Centre, McGill University, 3655 Sir William-Osler, Room 715, Montreal, Quebec, H3G1Y6 Canada

Accepted 23 July 2004
Available online 11 November 2004

Abstract

Tyrosine phosphorylation is negatively regulated by the protein-tyrosine phosphatases (PTPs). In order to find the physiological substrates of these enzymes, diverse PTP mutants that do not possess any catalytic activities but appear to bind tightly to their tyrosine phosphorylated substrates have been designed. Hence, they can be used as tools to pull out their respective substrates from heterogeneous extracts. Named PTP "substrate-trapping" mutants by the Tonks laboratory, they represent a diverse variety of defective PTPs that are epitomized by the Cys to Ser mutant (C/S) where the active cysteine residue of the signature motif is mutated to a serine residue. In addition, new mutants have been developed which are expected to help characterize novel and less abundant substrates. In this article, we review and describe all the different substrate-trapping mutants that have successfully been used or that hold interesting promises. We present their methodology to identify substrates in vivo (co-immunoprecipitation) and in vitro (GST pulldown), and provide a current list of substrates that have been identified using these technologies.

Keywords: Substrate-trapping; PTP; Protein-tyrosine phosphatase; PTP1B; GST pulldown

1. Introduction

Protein-tyrosine phosphorylation is a reversible post-translational modification that is essential for eukaryotic cells. The counteracting activities of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) regulate the level of cellular tyrosine phosphorylation. Because of the complexity of these gene families, to decipher the signaling in which each of the kinases and phosphatases are involved can be very challenging. One obvious step is to characterize their physiological substrates. Intuitively, it seems easier to find a substrate for a kinase than for a phosphatase. Kinases will act directly on their targets, which results on the addition of a phosphate group that can directly point to the substrates of the kinases (radioactively or using chemiluminescence). However, to uncover the phosphatase's substrates, one requires the detection of such phosphate removal from previously phosphorylated proteins. Furthermore, the promiscuous in vitro activity of PTP complicates the identification of genuine PTP substrates. Fortunately, the PTP field obtained a valuable tool with the generation of mutant PTPs that could act as substrate-trapping mutants.

Typically, in PTP substrate-trapping mutants, the ongoing PTP catalysis is blocked. As a consequence, the substrate is trapped in the catalytic pocket of the PTP. Such enzyme–substrate interaction is sufficiently stable so that the complex can be purified. Substrate-trapping mutants have been largely used to characterize (or confirm) physiological substrates and consequently the signaling pathway in which a PTP is involved. These mutants became a very useful and important biochemical tool. The characteristic of a good substrate-trapping mutant are (i) to be inactive or barely active (lowest k_{cat}),

* Corresponding author.
E-mail address: christophe.blanchetot@mcgill.ca (C. Blanchetot).

1046-2023/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2004.07.007

(ii) to bind efficiently to its physiological substrate (low k_m), and (iii) to keep its structural integrity as much as possible.

The PTP substrate-trapping mutants have been largely used in different set up. They have been used to find PTP specificity (this issue, Espanel et al.), to isolate specific inhibitor (this issue, Kumar et al.) and to screen cDNA library in a modified two-hybrid system (this issue, Fukada et al.). In this article, we review and describe all the different substrate-trapping mutants that have successfully been used or that hold interesting promises. We present the substrate-trapping methodology for in vivo (co-immunoprecipitation) and in vitro (pulldown) studies. We also listed the substrates that have been identified using these technologies.

2. Substrate-trapping mutants

The development of substrate-trapping mutants is a direct consequence of understanding the PTP's catalytic mechanism. Enzymatic and structural characterization of PTP1B, in particular, pinpointed to critical residues for phosphotyrosine catalysis whose mutations would generate a defective enzyme that could fulfill the criteria required to generate a substrate-trapping mutant. Experimentally, the substrate-trapping mutants the most used so far have been the mutants in which the signature motif cysteine was replaced by a serine (C/S), or in which the aspartate in the so-called "WPD loop" was mutated to alanine (D/A). However, other mutations have also been developed (sometimes in concert with the C/S or D/A) that improved this technical approach.

2.1. Cysteine to serine (C/S) mutants

The first substrate-trapping mutant described is the semi-conservative mutation of the catalytic cysteine to serine (C/S) [1–3]. The catalytic cysteine is essential and is found within the classical "PTP-signature motif" (I/V)H*C*SAGxxR(S/T)G. This cysteine (215 in PTP1B) has an extremely low pK_a (4.5–5.5) and is unprotonated at intracellular pH. The unprotonated cysteine acts as a nucleophile, attacking the phosphorus center of the substrate, leading to a phosphoryl–cysteine intermediate (PTP–Cys–PO_3) and further release of the dephosphorylated substrate (S). Replacement of the catalytic site cysteine by a serine allows binding of the physiological substrate to the mutant PTP but blocks the catalysis and a PTP–Ser–PO_3–S complex is formed which leads to the stabilization of the enzyme–substrate interaction. The C/S are catalytically dead, and are commonly used to isolate physiological substrates of PTPs (see Table 1).

2.2. Aspartate to alanine (D/A) mutants

Following substrate binding into the catalytic pocket, a very important structural change occurs. The tip of the loop that contains the highly conserved WPD motif (so-called "WPD" loop) flips over the phosphotyrosine residue. The movement is of approximately 8–12 Å length. The loop contributes to the hydrophobic pocket that buries the phosphotyrosine, holding the substrate into place and brings the critical aspartate residue (WP*D*) close to the phosphoryl–cysteine intermediate. The aspartate (D181 in PTP1B) serves at first as a general acid by protonating the leaving phenolic oxygen group from the substrate (P–O bond), whose event favors the expulsion of the dephosphorylated substrate from the catalytic site. The same aspartate is then postulated to serve as general base by reacting with a water molecule that will attack the Cys–PO_3 intermediate, liberating free phosphate and regenerating the enzyme active. The D/A mutation acts as a substrate-trapping mutant first, because of the absence of the aspartate acid role and second, through the flipping of the WPD loop that comes over the substrate and blocks it into the catalytic pocket, preventing its release. Mutation of the aspartate to alanine (D/A) also blocks the catalytic process and the PTP–D/A is almost completely catalytically inactive. In vitro, PTP1B–D181A k_{cat} is so reduced that its activity would be around 1 catalytic cycle per hour [4]. However, the D/A mutant of VHR and SHP2 were shown to conserve some activity in vivo [5]. The essential role of the aspartate is apparently partially taken by the hydroxy amino acid threonine, immediately C-terminal of the Arg in the VHC$(X)_5$R*T* motif, which facilitate the hydrolysis of the thiol ester intermediate [6]. The D/A mutation is probably the best substrate-trapping mutant used widely to date (Table 1). For most PTP tested, the substrate binding to the PTP–D/A is better than to the PTP–C/S mutants, although this was not the case for RPTPα and SHP2 [5,7]. The reason for such differences is not clear. The sensitivity to oxidation (during the purification or the pulldown itself) of the catalytic cysteine in the RPTPα-D/A or SHP2-D/A might be in cause, some phosphatases (domains) being more susceptible than other [8,9].

Interestingly, an adapted variant of the D/A mutant was used in the case of PTPH1. PTPH1–D811A was a good substrate-trapping mutant in vitro but not in vivo [10]. The very conserved tyrosine in the KNR*Y* motif (Y676 in PTPH1) was mutated to phenylalanine. Mutation of Y676 in the PTPH1–D811A dramatically reduced its tyrosine phosphorylation in vivo. The authors suggest that Y676 may have become a receptor for the phosphate from the highly active phosphoryl–cysteine intermediate (PTP–Cys–PO_3) in PTPH1–D811A. Tyrosine phosphorylation of Y676, most presumably impeded the access of any substrate to the