

The *Yersinia* Tyrosine Phosphatase: Specificity of a Bacterial Virulence Determinant for Phosphoproteins in the J774A.1 Macrophage

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Summary

YopH is a plasmid-encoded protein tyrosine phosphatase (PTPase) secreted by pathogenic *Yersinia*. Although the enzyme likely acts to dephosphorylate eukaryotic proteins during *Yersinia* infection of the mammalian host, the targets of YopH have not been identified. We infected the murine macrophage-like cell line J774A.1 with *Yersinia pseudotuberculosis* and investigated the specificity of YopH and YopHC403A, a catalytically inactive mutant derivative, for eukaryotic phosphoproteins. Upon infection, YopH specifically and rapidly dephosphorylated a macrophage protein of 120 kD. The 120-kD protein and a previously detected 55-kD substrate of YopH coprecipitated with YopHC403A. Coprecipitation of these proteins required tyrosine phosphorylation and could be competitively inhibited with excess phosphotyrosine. The 120- and 55-kD proteins that coprecipitate with YopHC403A exhibited the in vitro activity of protein tyrosine kinases (PTKases), suggesting that YopH dephosphorylates activated tyrosine kinases in vivo.

Tyrosine phosphorylation is a key component of the signal transduction processes that control the growth and differentiation of eukaryotic cells (1). Cellular levels of tyrosine phosphate are modulated by the concerted action of protein tyrosine kinase (PTKase)¹ and protein tyrosine phosphatase (PTPase) activities (2). Altering levels of tyrosine phosphorylation in the mammalian host is of fundamental importance for the virulence of two microbial pathogens that encode PTPases, vaccinia virus (3) and the pathogenic bacteria of the genus *Yersinia* (4, 5). The pathogenic *Yersinia* infect both animals and humans, share a marked predilection for the reticuloendothelial system of the host, and cause disease ranging from mild gastroenteritis and mesenteric lymphadenitis (*Y. enterocolitica* and *Y. pseudotuberculosis*) to bubonic plague (*Y. pestis*) (6).

Attachment and entry of *Y. pseudotuberculosis* into mammalian cells is mediated by invasin (7), and the *Yersinia* adhesin (Yad) (Bliska and Falkow, manuscript in preparation). These proteins recognize multiple members of the $\beta 1$ integrin family of extracellular matrix receptors (8). In addition, the capacity of these bacteria to proliferate extracellularly in the hostile

environment of the mammalian reticuloendothelial system is associated with the expression of a set of plasmid-encoded secreted proteins called Yops that are essential determinants of the bacterium's virulence (9). Yops are coordinately synthesized and released by *Yersinia* grown at 37°C in low calcium (Ca²⁺) medium (10). Several of the regulatory and secretory proteins required for Yop expression are homologous to proteins found in other prokaryotes (11, 12). YopH and YopM contain functional homology to eukaryotic signal transduction proteins, a finding which has led to the assignment of their molecular functions (4, 13).

The 51-kD YopH protein, encoded by the virulence plasmid pIB1, contains functional homology to the conserved catalytic domains of the PTPase family (4). The role of PTPase activity in *Yersinia* pathogenesis was demonstrated by changing an essential cysteine residue to an alanine residue in the catalytic domain of the protein (see Fig. 1). This single amino acid change abolished YopH PTPase activity and significantly reduced the virulence of *Y. pseudotuberculosis* in a murine infection model (5). Mutational inactivation of YopH activity either by the C403A codon substitution (our unpublished observations) or by deletion of *yopH* (14), has been shown to reduce the invading microbe's ability to resist phagocytosis by murine macrophages. This observation suggests that the interruption of the phagocytic process by YopH-mediated dephosphorylation of host proteins is a major pathogenic

¹ Abbreviations used in this paper: GST, glutathione-S-transferase; PDGF, platelet-derived growth factor; PTKase, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PTyr, O-phospho-L-tyrosine; Yad, *Yersinia* adhesin.

strategy of *Yersinia*. Consistent with this model was the demonstration that YopH specifically dephosphorylated proteins of 120 and 55 kD (previously termed 60 kD) in J774A.1 macrophage-like cells infected with *Y. pseudotuberculosis* (5). To better understand the effect of YopH on host cell function, we have sought to determine the identity and function of the 120- and 55-kD substrates. Here we show that the 120- and 55-kD proteins that are dephosphorylated by YopH in vivo are coprecipitated with YopHC403A and exhibit the in vitro activity of PTKases. These findings raise the possibility that YopH acts to subvert signal transduction within host cells by reversing the phosphorylation state of host PTKase molecules.

Materials and Methods

Bacterial Growth and Tissue Culture Infection Conditions. The strains of *Y. pseudotuberculosis* used and their growth conditions have previously been described (5). J774A.1 cells were grown and prepared for infection assays as described (5). Before infection, the bacteria were pregrown to mid-log phase at 37°C in Lennox base broth (Gibco BRL, Gaithersburg, MD). Monolayers of J774A.1 cells (2×10^6 cells) in 60×15 mm dishes were placed on ice and overlaid with ice-cold serum-free DMEM supplemented with 25 mM Hepes, pH 7.4 and 0.4% BSA. Bacteria (2×10^7 CFUs) were inoculated onto the monolayers, and the dishes were briefly centrifuged at 4°C (7). After allowing for the bacteria to associate with the cells on ice for 90 min, the media overlaying the monolayers was replaced with media prewarmed to 37°C. Before the temperature upshift, or at various times thereafter, the cultures were lysed on ice with 0.25 ml lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 0.2 mM NaVO₄, 10 mM NaF, 0.4 mM EDTA) as described (5).

Antibodies. The antiphosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal rabbit antibody specific for the platelet-derived growth factor (PDGF) receptor was supplied by Oncogene Science, Inc. (Manhasset, NY). The RAY51 antibody was raised against recombinant Yop51, a homolog of YopH expressed by *Y. enterocolitica* (15). The Yop51 protein was purified as a glutathione-S-transferase (GST) fusion by affinity binding to glutathione agarose (Sigma Chemical Co., St. Louis, MO) using a procedure similar to that previously described (16). Yop51 was subsequently isolated from the GST domain by thrombin (Sigma Chemical Co.) cleavage. Polyclonal RAY51 antibodies were raised by a series of three monthly injections of female New Zealand white rabbits with 200 µg Yop51 in adjuvant (Ribi ImmunoChem Research Inc., Hamilton, MT). Antisera was collected and affinity purified on a column consisting of Yop51 coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, CA) using a procedure as described (17).

Immunoprecipitations, Electrophoresis, and Immunoblotting Techniques. Proteins were precipitated from the clarified lysates by the addition of 1 µg of 4G10, RAY51, or polyclonal rabbit anti-PDGF receptor as described (5). Where indicated, *O*-phospho-L-tyrosine (Sigma Chemical Co.) was added (10 mM) to the lysate before immunoprecipitation. Immune complexes and prestained protein molecular weight standards (Gibco BRL) were displayed by electrophoresis on 8–15% polyacrylamide SDS gels and electrophoretically transferred to nitrocellulose filters (17). The immunoblotting techniques have been described (17). Immunoblotting with the 4G10 antibody was as specified by the supplier. The RAY51 antibody was used at a concentration of 1 µg/ml. Antibody binding was

detected with secondary anti-mouse or anti-rabbit antibodies conjugated to alkaline phosphatase (Sigma Chemical Co.) and (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) reagents purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Kinase Assay and Phosphoamino Acid Analysis. Immune complexes were washed three times (1 ml each) with kinase assay buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 0.05% BSA), and incubated in 10 µl of kinase assay buffer containing 60 µM ATP and 5 µCi γ-[³²P] ATP (Amersham Corp., Arlington Heights, IL) for 30 min at 37°C. The reaction products and ¹⁴C-labeled protein molecular weight standards (Amersham Corp.) were resolved by electrophoresis on 8–15% polyacrylamide SDS gels, transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories), and labeled proteins were visualized by exposure of the filter to x-ray film at –70°C for 3 h. The portions of the PVDF filter containing the immobilized 120- and 55-kD phosphoproteins were excised and subjected to phosphoamino acid analysis as described (18).

Results

We have previously demonstrated that within 3 h, two constitutively tyrosine-phosphorylated macrophage proteins of 120 and 55 kD are specifically dephosphorylated by YopH in J774A.1 cells infected with *Y. pseudotuberculosis* (5). To better characterize these J774A.1 substrates, and to determine the kinetics of dephosphorylation, an antiphosphotyrosine mAb was used in conjunction with immunoblotting techniques to detect the effect of YopH on host tyrosine phosphorylation.

The appearance of tyrosine-phosphorylated proteins in J774A.1 cells over a time course infection is demonstrated in Fig. 2. Cell monolayers were infected on ice with strains of *Y. pseudotuberculosis* to permit the bacteria to attach to host receptors (7) while preventing functional expression of the bacterial PTPase. At time zero, the cultures were warmed to 37°C and at various times thereafter detergent lysates were prepared in the presence of phosphatase inhibitors. Tyrosine-phosphorylated proteins in the lysates were detected by immunoblotting with the antiphosphotyrosine mAb 4G10 (19). Several alterations in tyrosine phosphorylation were evident over the time course of infection. Dephosphorylation of a major 120-kD protein was specific to the bacterial infection

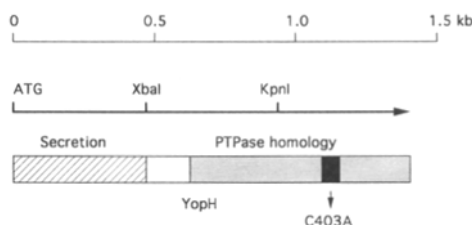


Figure 1. Structure of the YopH protein and position of the catalytic mutation in YopHC403A. Shown is a representation of the *yopH* gene and its protein product. (▨) Catalytic domain homologous to eukaryotic PTPases. (■) Catalytic site containing the conserved cysteine (Cys) residue which was converted to alanine (Ala) at position 403 in YopHC403A. (▧) NH₂-terminal domain involved in secretion of the protein by the bacteria (28).

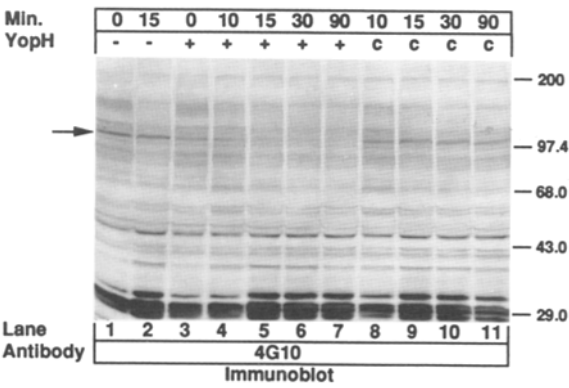


Figure 2. Time course infection assay of J774A.1 cells with *Y. pseudotuberculosis*. Strains of *Y. pseudotuberculosis* YPIII were pregrown at 37°C and inoculated onto monolayers of J774A.1 cells on ice. After allowing for bacterial binding, the cultures were warmed to 37°C, and at the times indicated, detergent lysates were prepared and analyzed by immunoblotting with the antiphosphotyrosine mAb 4G10. Samples shown were from uninfected macrophages (-) (lanes 1 and 2), macrophages infected with wild-type YPIIIpIB1 (+) (lanes 3-7), and macrophages infected with the catalytic mutant YPIIIpIB1C403A (c) (lanes 8-11). (→) Position of the 120-kD substrate. Sizes of the molecular weight standards in kD are shown.

(lanes 3-11), whereas the other changes were related to the temperature shift, as these alterations occurred in uninfected cells (lanes 1 and 2). Dephosphorylation of the 120-kD substrate began rapidly, within 15 min of the temperature up-shift (lane 5), and was complete by 90 min (lane 7). Dephos-

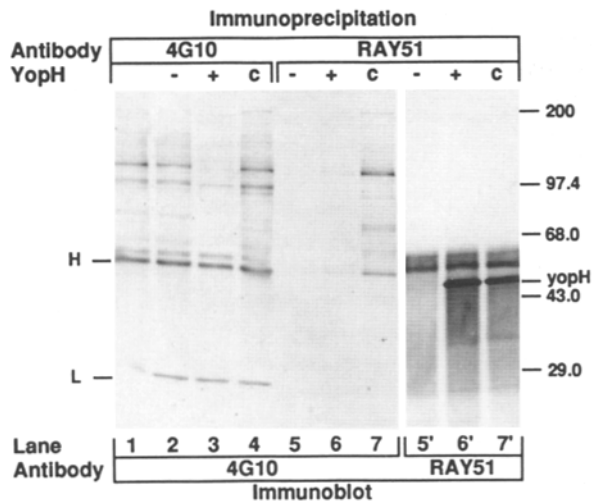


Figure 3. Binding and dephosphorylation of macrophage proteins by the *Yersinia* PTase. J774A.1 cells were left uninfected (lane 1), or infected with plasmid-cured YPIII (-) (lanes 2, 5, and 5'), wild-type YPIIIpIB1 (+) (lanes 3, 6, and 6'), or mutant YPIIIpIB1C403A (c) (lanes 4, 7, and 7') for 180 min. Detergent lysates were prepared and immunoprecipitated with 4G10 (lanes 1-4) or RAY51, an affinity-purified rabbit polyclonal antibody specific for YopH (lanes 5-7'). The immune complexes were analyzed by immunoblotting with the 4G10 antibody (lanes 1-7) or the RAY51 antibody (lanes 5'-7'). Equal portions of the same samples were analyzed in lanes 5-7 and 5'-7'. Positions of the prestained molecular weight markers, the heavy (H) and light (L) chains of the precipitation antibodies and YopH are indicated.

phorylation was specific to YopH. The 120-kD protein was not dephosphorylated in cells infected with bacteria expressing YopHC403A (lanes 8-11). As shown below, the 55-kD substrate is also present in these cells, but was not detected in the experiment shown in Fig. 2, presumably because it comigrated with more abundant phosphoproteins.

Although YopHC403A is catalytically inactive, it is functionally expressed by the bacteria (5), and we presume, must be introduced normally into the macrophage cell. Therefore, we looked for a possible interaction between YopHC403A and the substrates of YopH. Detergent lysates from infected macrophages were prepared and immunoprecipitated with an affinity-purified rabbit polyclonal antibody (RAY51) that recognizes YopH. The immune complexes were analyzed by immunoblotting with the 4G10 or RAY51 antibodies (Fig. 3). Multiple phosphoproteins were coprecipitated with the RAY51-YopHC403A complex (lanes 7 and 7'). At a reduced level, these proteins were also found in the RAY51-YopH complexes (lanes 6 and 6'). Coprecipitation of these proteins was dependent on the presence of the bacterial PTPase (lanes 5 and 5'). For reference, proteins immunoprecipitated with the 4G10 antibody were analyzed in parallel (lanes 1-4). The 120-kD YopHC403A-associated protein appeared to be identical to the 120-kD protein dephosphorylated by YopH (lane 3). Because the 55-kD YopHC403A-associated protein (lane 7) comigrated with the H chain of the precipitation antibody, in this assay we were unable to show that it was dephosphorylated by YopH (lane 3). In other experiments in which ³²P-labeled immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography, we confirmed that the 55-kD-YopHC403A-associated protein comigrated with the 55-kD substrate (data not shown).

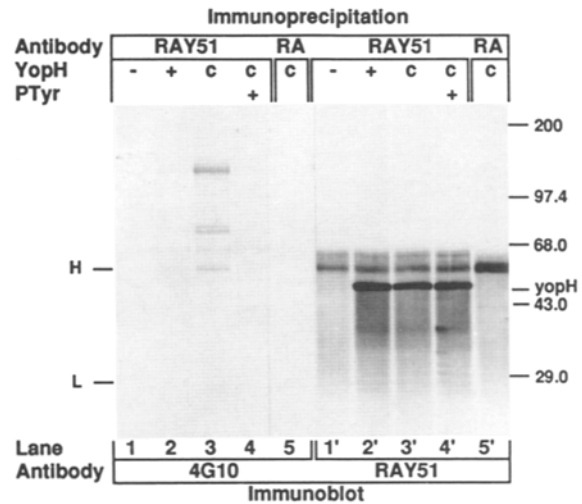


Figure 4. Inhibition of YopHC403A substrate binding by phosphotyrosine. J774A.1 cells were infected as described in Fig. 3 and proteins were immunoprecipitated with RAY51 (lanes 1-4 and 1'-4') or a control antibody (RA, polyclonal rabbit anti-PDGF receptor) (lanes 5 and 5'). Before immunoprecipitation, O-phospho-L-tyrosine (PTyr) was added (10 mM) to the lysate analyzed in lanes 4 and 4'. Equal portions of each immunoprecipitated sample was analyzed by immunoblotting with 4G10 (lanes 1-5) or RAY51 (lanes 1'-5') antibodies.

The association of these proteins with YopHC403A might reflect the affinity of the PTPase for tyrosine-phosphorylated substrates. To investigate the importance of tyrosine phosphorylation in YopHC403A-substrate binding, we tested whether or not binding could be competitively inhibited with excess phosphotyrosine. In the presence of 10 mM phosphotyrosine, the binding of the substrates to YopHC403A was completely inhibited (Fig. 4, lanes 3 and 4). Phosphotyrosine did not affect the binding of YopHC403A to the antibody (lanes 3' and 4').

A previous observation that the 120- and 55-kD substrates were constitutively phosphorylated on tyrosine (5) in the transformed J774A.1 cell line (20), led us to speculate that these proteins might be activated PTKases. Therefore, we incubated immunoprecipitated complexes from infected macrophages in an in vitro kinase reaction, and analyzed the ^{32}P -labeled products by SDS-PAGE and autoradiography (Fig. 5). Significant phosphorylation of the 120- and 55-kD proteins bound to YopHC403A was evident (lane 2). This pattern of labeling was similar to the labeled products of a 4G10 immune complex kinase assay (lane 1). The specificities of the kinase activities were determined by phosphoamino acid analysis of the 120- and 55-kD phosphoproteins. Both proteins were found to be phosphorylated preferentially on tyrosine (Fig. 6). This result is consistent with the idea that the 120- and 55-kD proteins dephosphorylated by YopH are in fact autophosphorylated PTKases that are constitutively activated in the J774A.1 cell.

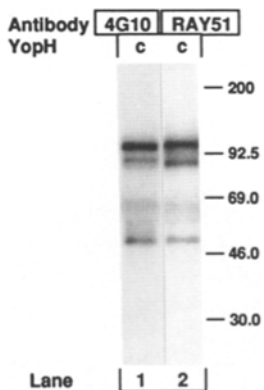


Figure 5. In vitro kinase activity associated with YopHC403A. Detergent lysates were prepared from macrophages infected with YPIIIpB1C403A and proteins were immunoprecipitated with 4G10 (lane 1) or RAY51 (lane 2) after the standard protocol. The immune complexes were incubated in an in vitro kinase reaction and the ^{32}P -labeled products were resolved by SDS-PAGE, transferred electrophoretically to PVDF membrane, and labeled proteins were visualized by exposure of the filter to x-ray film. The positions of the ^{14}C -labeled molecular weight markers are indicated.

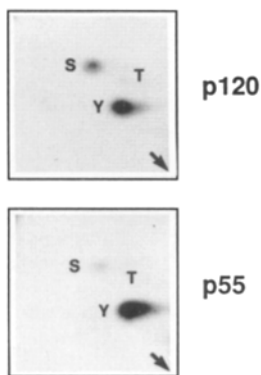


Figure 6. Phosphoamino acid analysis of the in vitro kinase reaction products. The portions of the PVDF filter containing the immobilized 120- and 55-kD phosphoproteins (Fig. 5, lane 2) were excised and subjected to phosphoamino acid analysis. (→) Approximate origins. Positions of the ninhydrin stained markers are labeled (S) phosphoserine, (T) phosphothreonine, and (Y) phosphotyrosine.

Discussion

To initiate oral infection of a mammalian host, the enteropathogenic *Yersinia* cross the intestinal epithelium to reside in a specialized lymphoid follicle known as the Peyer's patch (21). As part of the normal Peyer's patch function as an outpost of the host reticuloendothelial system, microbes, and other antigens from the bowel lumen are presented by the specialized M cell to lymphocytes and macrophages that patrol the underlying Peyer's patch. Because pathogenic strains of *Y. pseudotuberculosis* are able to grow and proliferate in this hostile environment, key targets of YopH likely include signal transduction processes in phagocytic and other immune cells. It was with this goal in mind that we initiated our studies to define the effect of YopH on cells involved in host resistance to bacterial infection using the J774A.1 macrophage-like cell line as a tissue culture infection model.

Our results demonstrate that two tyrosine-phosphorylated proteins of 120 and 55 kD are the primary targets of YopH in J774A.1 cells. In a time course infection assay in which functional expression of YopH was highly synchronized, we found that the 120-kD substrate was rapidly and efficiently dephosphorylated by the bacteria. The speed of this dephosphorylation event correlated well with the short time interval (<30 min) in which the physiological effect of YopH on the macrophage phagocytic process can be detected (14) (our unpublished observations).

In ^{32}P -orthophosphate labeling experiments, the 120- and 55-kD proteins were initially detected as constitutively and highly tyrosine-phosphorylated components of the J774A.1 cell (5). We believed that these proteins were activated PTKases that were constitutively autophosphorylated. Our results are consistent with this model, as we have shown that both proteins are tyrosine phosphorylated in vitro. It is important to note that we have not ruled out the possibility that the 120- and 55-kD proteins are substrates of an unidentified PTKase bound to YopHC403A.

Activation of receptor PTKases, which involves autophosphorylation, is the first step in the process of transducing an extracellular signal to a cellular response (1). Eukaryotic PTPases are thought to be capable of modulating this event, and their activity may in large part be controlled by cellular localization (2). It is tempting to speculate that YopH acts as an unregulated PTPase that can reverse the activation of PTKases. There is precedence for a model in which YopH inhibits the phagocytic capability of macrophages through its effect on PTKase function. Uptake of *Yersinia* into mammalian cells is mediated by integrin receptors (8) (Bliska and Falkow, manuscript in preparation). In fibroblasts, the uptake process can be blocked by inhibitors of PTKase activity (22), and $\beta 1$ integrin crosslinking stimulates tyrosine phosphorylation of a 125-kD focal adhesion PTKase (23, 24). However, attempts to identify the 120- and 55-kD proteins with antibodies has thus far failed. Two likely candidates we tested included the 125-kD focal adhesion PTKase (23), and a 55-kD *Src* homolog called *Fgr* that is expressed in hematopoietic lineages (25). Based on the affinity of the 120- and 55-kD proteins for YopHC403A, we have therefore begun to purify

sufficient quantities of these potentially novel PTKases for sequencing studies and antibody production.

The binding of tyrosine-phosphorylated proteins by YopHC403A is reminiscent of the interaction of *Src* homology 2 (SH2) domains of eukaryotic signal transduction proteins with autophosphorylated PTKases (26). The phosphotyrosyl binding activity of the SH2 domain is thought to promote recruitment of cytoplasmic signaling proteins to activated PTKases to facilitate the transduction of external signals to second messenger pathways. Although the role of protein sequence in specifying the binding of substrates by YopH has not been addressed here, sequence motifs are known to be important for SH2-mediated interactions (26). The association of YopHC403A and SH2 domains with their respective tyrosine-phosphorylated substrates is likely to be mechanistically different, since the binding domains of these proteins do not share significant homology.

A major impediment to the elucidation of PTKase func-

tion has been the difficulty with which physiologically relevant substrates have been identified (1). Our demonstration that the substrates of YopH can be detected and characterized by virtue of their association with a catalytically inactive form of the protein has been potentially important implications for the molecular analysis of other PTPases. Although the substrate specificity of YopH appears to be broad, other PTPases are thought to be highly specific. One example is CDC25, which acts preferentially on a complex of CDC2 and cyclin (27). By creating catalytically inactive forms of eukaryotic PTPases, it should be possible to purify and identify substrates by virtue of their affinity for the enzyme. An extension of this approach could be the use of catalytically inactive PTPases as highly specific biochemical or cytological probes to detect the presence of tyrosine phosphate in substrates during the growth and differentiation phases of eukaryotic cells.

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