A Single Mutation Converts a Novel Phosphotyrosine Binding Domain into a Dual-specificity Phosphatase*

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Matthew J. Wishart, J ohn M. Denu,§ J ohn A. Williams†, and Jack E. Dixon‡
From the Departments of Physiology, Biological Chemistry, and N ranal Medicine, The University of Michigan, Ann Arbor, Michigan 48109-0606

Dual-specificity protein-tyrosine phosphatases (ds-PTPases) have been implicated in the inactivation of mitogen-activated protein kinases (MAPKs). We have identified a novel phosphoserine/threonine/tyrosine-binding protein (STYX) that is related in amino acid sequence to ds-PTPases, except for the substitution of Gly for Cys in the conserved dsPTPase catalytic loop (HC/XXGXXXR/S/T). cDNA subcloning and Northern blot analysis in mouse reveals pol(A') hybridization bands of 4.6, 2.4, 1.5, and 1.2 kilobases, with highest abundance in skeletal muscle, testis, and heart. Polymerase chain reaction amplification of reverse-transcribed poly(A') RNA revealed an alternatively spliced form of STYX containing a unique carboxyl terminus. Bacterially expressed STYX is incapable of hydrolyzing Tyr(P)-containing substrates; however, mutation of Gly120 to Cys (G120C), which structurally mimics the active site of ds-PTPases, confers phosphatase activity to this molecule. STYX-G120C mutant hydrolyzes p-nitrophenyl phosphate and dephosphorylates both Tyr(P) and Thr(P) residues of peptide sequences of MAPK homologues. The kinetic parameters of dephosphorylation are similar to human ds-PTPase, Vaccinia H1-related, including inhibition by vanadate. We believe this is the first example of a naturally occurring “dominant negative” phosphoserine/threonine-serine/threonine-binding protein which is structurally related to ds-PTPases.

Dual-specificity phosphatases (ds-PTPase) hydrolyze phosphoserine/threonine/tyrosine-containing substrates in vitro and exhibit a substrate preference in vitro and in vivo for diphosphorylated (Thr(P)/Tyr(P)) mitogen-activated protein kinase (MAPK) homologues (reviewed in Ref. 1). Most ds-PTPases are localized to the nucleus (2–4), and it has been suggested that they are responsible for the nuclear dephosphorylation and activation of MAPKs seen in vivo (5). All ds-PTPases contain the sequence, H/CXXGXXXR/S/T, which has been shown to correspond to the active site of PTPases (6). The essential cysteine forms a thiolphosphate intermediate during ds-PTPase-catalyzed dephosphorylation (7, 8). Several investigators have shown that substitution of this Cys, by Ser, in the dsPTPases, abolishes hydrolytic activity in vitro (7, 9–12) and function in vivo (10, 13, 14). Interestingly, transient expression of the Cys to Ser mutant prolongs MAPK activation in vivo (12), suggesting that it may compete with native phosphatases for binding to phosphorylated MAPK. Moreover, the mutant dsPTPase co-immunoprecipitates with phosphorylated MAPK (10), indicating that the catalytic cysteine is not essential for substrate binding. This conclusion is also supported by PTPase crystallographic studies showing that Cys to Ser mutants bind sulfate (15) and tyrosine-phosphorylated peptide (16) in a manner identical with native enzymes.

This paper describes the identification and isolation of a novel phosphoserine/threonine/tyrosine-binding protein (STYX) that is related in amino acid sequence to ds-PTPases, but contains a naturally occurring Gly residue in place of the active site, catalytic Cys. Residues which have been shown to be important in binding of phosphorylated substrates by ds-PTPases are present in STYX. Expression of recombinant STYX showed that it had no phosphatase activity; however, a single mutation of Gly120 to Cys (STYX-G120C) confers phosphatase activity to the recombinant mutant protein. The STYX-G120C mutant had binding constants and kinetic parameters similar to ds-PTPases. We believe this is the first example of a naturally occurring “dominant negative” phosphoserine/threonine-tyrosine-binding protein, structurally related to ds-PTPases.

EXPERIMENTAL PROCEDURES

PCR Subcloning and Northern Blot Analysis—Mouse testis poly(A+) RNA was extracted from adult tissue using TriZOL (Life Technologies, Inc.), followed by PolyATtract (Promega) purification according to manufacturer’s instructions. First strand cDNA synthesis was carried out with oligo(dT) and random primers using the cDNA Cycle Kit (Invitrogen), and DNA amplification was performed by polymerase chain reaction (PCR) with primers to mouse testis expressed sequence tag F220A (GenBank accession number L26737). Fifty pmol each of 5'-primer (5'-GATATCTGGTCAATCACCCAGAAC-3') and 3'-primer (5'-GATATCTGGTCAATCACCCAGAAC-3') were used with Taq polymerase (Boehringer Mannheim) for 35 cycles of 94 °C, 2 min; 50 °C, 2 min; 72 °C, 3 min. The single PCR product of 314 bp was ligated into pCR™ II plasmid (Invitrogen), and DNA amplification was performed by polymerase chain reaction (PCR) with primers to musle testis expressed sequence tag F220A (GenBank accession number L26737). Fifty pmol each of 5'-primer (5'-GATATCTGGTCAATCACCCAGAAC-3') and 3'-primer (5'-GATATCTGGTCAATCACCCAGAAC-3') were used with Taq polymerase (Boehringer Mannheim) for 35 cycles of 94 °C, 2 min; 50 °C, 2 min; 72 °C, 3 min. The single PCR product of 314 bp was ligated into pCR™ II plasmid (Invitrogen), and sequencing by dyeoxy chain termination using Sequenase II polymerase (U. S. Biochemical Corp.). The 314-bp insert of the pCR™ II subclone (TA314) was used as a template for probe synthesis by PCR as above, using [α-32P]dATP in the reaction. 32P-labeled PCR product was used to probe a mouse adult multiple tissue poly(A+) Northern blot (Clontech) at ~2 × 10^6 cpm/ml under manufacturer’s conditions. Final washing was performed at 50 °C in 0.1 × SSC (1 × SSC is 150 mM NaCl, 15 mM NPPp, p-nitrophenyl phosphate; MAPK, mitogen-activated protein kinase; bp, base pair(s); HPLC, high performance liquid chromatography.

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† To whom correspondence should be addressed: Dept. of Biological Chemistry, University of Michigan Medical School, Rm. 5416 Medical Science I, Ann Arbor, MI 48109-0606. Tel.: 313-764-8192; Fax: 313-763-4581.

‡ The abbreviations used are: ds-PTPase, dual-specificity protein-tyrosine phosphatase; Tyr(P), phosphorysine-threonine/tyrosine; VHR, Vaccinia H1-related; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane; Bis-Tris, bis(2-hydroxyethyl)iminotrismethoxymethane.
NAD, citrate, pH 7.0. 0.1% SDS for 40 min, and hybridizing bands were visualized by autoradiography at −80°C with intensifying screens. Washing at 65°C for 60 min had no effect on either the tissue distribution or number of bands seen. Subclone TA228 was produced by PCR amplification of mouse testis cDNA described above, with 5′-primer (5′-TGGTCCCAACATCTCAGCTGGCGTGCT-3′) and 3′-primer (5′-AGCTACACCTCTATTTGGG-3′) for 35 cycles of 94°C, 2 min; 47°C, 2 min; 72°C, 3 min, followed by ligation and sequencing of the 228-bp product as above.

Library Screening—Approximately 10^6 subclones were screened from both a Lambda Max1 mouse testis 5′-stretch cDNA library (Clontech) and Uni-Zap XR mouse diaphragm cDNA library (Stratagene) per manufacturer’s instructions. Subclone TA314 was used for probe synthesis by PCR as described above. Finally, washing of nitrocellulose filters was performed at 65°C in 0.2 × SSC, 0.1% SDS for 60 min. Positive subclones were identified by autoradiography and plaque-purified. Phagemids were excised and rescued from purified phage according to the manufacturer. Stratagene library phagemid, ST9, contained an insert of 1150 bp which overlapped the sequence of all the other phagemid clones isolated. All rescued phagemids were sequenced on both strands as above.

In Vitro Transcription/Translation—Phagemid ST9 was used as template for in vitro transcription/translation using [35S]Met and the TNT coupled Reticulocyte Lysate System (Promega), according to the manufacturer. Protein products were resolved by SDS-PAGE and identified by autoradiography without enhancement.

Overexpression and Purification of GST Fusion Proteins—Coding sequence contained within the 886-bp fragment of an NcoI/XhoI partial digest of ST9 was ligated into the NcoI/XhoI sites of bacterial expression plasmid, pGEX-KG (18), to produce pGEX-Native (NA) plasmid. Mutations of Gly to Ser were introduced by QuikChange site-directed mutagenesis of pGEX-NA plasmid, which was completely digested by NcoI and XhoI. The Gly substitution and were used in PCR reactions with pGEX-specific 5′- primer (kindly provided by Dr. G. Zhou) and mutated ST9 3′- primer (5′-ACTTCTAGAGATACCTGCATTRCAATG-3′) for 35 cycles of 94°C, 2 min; 45°C, 1.5 min; 72°C, 1.5 min. The 383-bp XbaI fragment of pGEX-NA was replaced with the corresponding XbaI fragment of the PCR reaction to produce pGEX-G120C. The identities of both pGEX constructs were confirmed by sequencing and differed only in the two nucleotides that convert Gly to Ser (GGG to GAA).

In Vitro Kinetic Assays—GST fusion proteins were separated from bacterial proteins by incubation with glutathione-agarose beads, washing, and elution off the beads in a buffer consisting of 0.05M Tris, 0.05 M Bis-Tris, and 0.1M acetate.

Kinetic Assays—Three different quantitative enzymatic assays were performed on GST-G120C, exactly as described for human dsPTPase, VHR (8). All assays were performed at 30°C, in a three-component buffer consisting of 0.05 M Tris, 0.05 M Bis-Tris, and 0.1 M sodium acetate. Hydrolysis of PTPase substrate, p-nitrophenyl-phosphate (pNPP), was followed as an increase in absorbance at 410 nm of the reaction product, p-nitrophenol. Initial rates at various initial substrate concentrations were fitted directly to the Michaelis-Menten equation using the nonlinear least squares program, Kinnetest for the Macintosh (IntelliKinetics, State College, PA). A continuous spectrophotometric assay was used to follow the Tyr(P) dephosphorylation of Tyr(P)/Thr(P)-containing peptides as described previously (8). The complete time course of the reaction was fitted to a modified integrated form of the Michaelis-Menten equation using a nonlinear least squares algorithm to obtain kinetic parameters kcat and Km. The third method involved reverse phase HPLC separation and quantitation of the substrates and products from the reaction of GST-PTPase with MAPK, LCK, and Epp1VATR (8). With this method, the rate of enzyme-catalyzed hydrolysis at Thr(P) was determined as above.

RESULTS AND DISCUSSION

Identification, PCR Subcloning, and Tissue Distribution—We identified an expressed sequence tag isolated from mouse testis (GenBank accession number L26737) that contained a partial open reading frame homologous to the active site of dual-specificity protein-tyrosine phosphatases (dsPTPases) (1). Several amino acids critical for phosphatase activity were conserved (8); however, there appeared to be a glycine substitution for the conserved cysteine within the active site of the putative phosphatase (Fig. 1A). Site-directed mutagenesis of the corresponding Cys, to Ser, has been shown previously to inactivate several dsPTPases catalytically (7, 9–14), while preserving substrate binding (10, 15, 16). Since it was possible that the Gly codon reflected errors in the original data base entry (i.e. a sequencing or PCR error), we initially attempted to confirm the sequence of the open reading frame. Primers were designed to specifically amplify a 314-bp sequence encompassing the Gly substitution and were used in PCR reactions with cDNA of reverse-transcribed mouse testis poly(A)′ RNA. As a consequence of the Gly substitution (Val-His-Gly), a unique NcoI site was created (5′-GTC_CAT_GGG-3′) which would not be found at the active site (Val-His-Cys) of PTPases (5′-GTN_CAY_TGY-3′). PCR reactions yielded a single product of 314 bp which was completely digested by NcoI (not shown), suggesting not only that the Gly codon substitution existed in the poly(A)′ RNA, but that a Cys-containing codon (i.e. catalytically active homologue) did not exist. The entire PCR reaction product was ligated into vector, and the sequence of 30 subclones all contained the glycine substitution (Fig. 1B). Similar results were obtained using mouse genomic DNA, reverse-transcribed mouse testis total RNA, and reverse-transcribed rat pancreas poly(A)′ RNA as templates for PCR (not shown), suggesting that the glycine codon did not arise from post-transcriptional modification and that its expression was not restricted to mouse. Hybridization of the 314-bp PCR product to mouse testis RNA, but that a Cys-containing codon (i.e. catalytically active homologue) did not exist. The entire PCR reaction product was ligated into vector, and the sequence of 30 subclones all contained the glycine substitution (Fig. 1B). Similar results were obtained using mouse genomic DNA, reverse-transcribed mouse testis total RNA, and reverse-transcribed rat pancreas poly(A)′ RNA as templates for PCR (not shown), suggesting that the glycine codon did not arise from post-transcriptional modification and that its expression was not restricted to mouse. Hybridization of the 314-bp PCR product to mouse testis poly(A)′ RNA demonstrated bands at 4.6, 2.4, 1.5 and 1.2 kb, with highest abundance in skeletal muscle, adrenal gland, and heart (Fig. 1C). Collectively, these observations suggested that the Gly codon was present in the expressed sequence tag, was also present in genomic DNA as well as poly(A)′ RNA, and that it was expressed in several tissues of both mouse and rat.

Isolation of cDNA Library Subclones—In an effort to obtain a full-length subclone, cDNA libraries from mouse testis and skeletal muscle were screened with the 314 bp PCR product described above. Several overlapping subclones were rescued and sequenced, with the longest clones from the two libraries (ST9 and CT20) schematically represented in Fig. 2A. Additional subclones were identified by PCR (TA228) and data base searches (EST2). All subclones contained identical nucleotide sequence in their 5′ ends (bases 1–759), including the Gly codon in the putative active site; however, the 3′ ends appeared to encode two distinct amino acid sequences. One subset of clones (EST1, TA314, and TA228) contained nucleotides 760–895 which code for the carboxyl terminus seen in the original expressed sequence tag. The remaining clones (CT20, EST2, and ST9) lacked these 136 nucleotides and consequently encoded a different carboxyl end. Interestingly, nucleotides 896–1273 were identical for all the subclones, suggesting that the
two forms were alternatively spliced products of the same mRNA (entire composite nucleotide sequence deposited as GenBank™ U34973). Moreover, examination of nucleotides 760–895 revealed a strong similarity to the consensus sequences of intron borders (see Ref. 19 for review), except for substitution of cytosine for guanine at the putative 5′ donor site (Fig. 2B). Substitution for guanine at this position has been shown to attenuate intron splicing efficiency (19) and is implicated in the retention of introns in mRNA from genes underlying disease states (20). Thus, the alternative splicing of this region could give rise to proteins with different carboxyl ends (Fig. 2A, Spliced and Unspliced), while preserving the glycine codon within the putative active site. Attempts to isolate the 136-bp “intron” from commercial cDNA libraries were unsuccessful; however, semiquantitative reverse transcription-PCR with poly(A)⁺ mouse testis RNA showed a relative abundance of spliced to unspliced product of approximately 100:1, respectively (not shown).

Cell-free Expression and Amino Acid Similarity to VHR—Since the size of the ST9 subclone (1.16 kb) approximately equaled the smallest poly(A)⁺ RNA species seen by Northern analysis (Fig. 1C), we examined the nature of the cell-free translation products expressed in a rabbit reticulocyte system. Protein products of -25 and 23 kDa (Fig. 3A) agreed very well with the predicted sizes (25.4 and 22.6 kDa) of proteins utilizing methionine codons at nucleotide positions 256–258 and 328–330 (GenBank™ U34973), respectively. Since both methionines reside in the same open reading frame, the upstream Met256–258 was designated residue +1 of an open reading frame coding for 223 amino acids (Fig. 3B). There remains the possibility that the ST9 sequence represents a partial open reading frame contained within longer mRNAs (Fig. 1C); however, additional support for Met256–258 as a translational start site comes from surrounding nucleotide similarity (5′-GGG ACC ATG G-3′) with the consensus sequence of vertebrate start sites (5′-GCC RCC ATG R-3′) (17). Met256–258 also correlates with the known start site of human dsPTPase, VHR (9) (Fig. 3B), which operationally defines the smallest PTPase domain.

We have named the sequence defined by the ST9-Met256–258 reading frame, STYX, for phosphoglycerine/threonine/tyrosine interaction protein. The putative alternative carboxyl terminus (Unspliced, Fig. 2A) is referred to as STNS, for alternatively spliced intron of STYX. Amino acid comparison of STYX with VHR revealed extensive sequence similarity (46.4%), including the conservation of VHR residues Asp92 and Arg130, previously shown to be important for substrate binding (8).

Bacterial Expression, Conversion, and Kinetics—The STYX coding sequence shown in Fig. 3B was ligated into pGEX-KG vector (18) and expressed as a GST-fusion protein. Purified GST-STYX fusion protein failed to show any hydrolytic activity toward the PTPase substrate, para-nitrophenyl phosphate (pNPP) (Fig. 4A). Since STYX contained all the structural elements thought to be important for dsPTPase activity (8), except the active-site Cys, we thought that replacing the naturally occurring Gly with Cys might restore catalytic activity. Mutation of Gly120 to Cys (STYX-G120C) conferred phosphatase activity to the fusion protein, as demonstrated by the hydrolysis of pNPP (Fig. 4A), with a kcat of 4.6 s⁻¹ and a Kₘ of 9.4 mM. Interestingly, preliminary studies showed that native STYX can inhibit the pNPP hydrolytic activity of the Cys-containing mutant.³ The dual-specific nature of the STYX-G120C mutant was demonstrated through its dephosphorylation of both Tyr(P) and Thr(P) of diphosphorylated MAP kinase peptide (Fig. 4B). Removal of GST via thrombin cleavage had no effect on the activity of either native STYX or the G120C mutant (not shown). Surprisingly, the kinetics of hydrolysis for STYX-G120C mutant were comparable to native

³ M. J. Wishart, personal observation.
Met-labeled proteins from cell-free STYX (performed as described under "Experimental Procedures" with native ric absorbance of product, translation below).

Fig. 3. Cell-free transcription/translation of STYX cDNA and amino acid alignment with dsPTPase, VHR. A, autoradiograph of [35S]Met-labeled proteins from cell-free in vitro transcription/translation of Stratagene subclone ST9 (Fig. 2A) resolved by SDS-PAGE. The position of molecular mass standards in kDa is shown at right. An arrowhead denotes the ~25-kDa protein corresponding to the open reading frame translation below. B, amino acid comparison of ST9-Met256–258 open reading frame (STYX) with dsPTPase, VHR (9). The alternative carboxy-terminal end contained in TA228 (Fig. 2A) is shown (STNS). Similar amino acids between STYX, STNS, and VHR are highlighted. Asterisks denote in-frame stop codons. An arrowhead marks the glycine substitution shown in Fig. 1A.

Fig. 4. Hydrolytic activity of native and mutant GST-STYX fusion proteins. A, hydrolysis of pNPP measured by spectrophotometric absorbance of product, para-nitrophenol, at 405 nm. Assays were performed as described under "Experimental Procedures" with native STYX (diamonds) and STYX-G120C (circles) fusion proteins at 480 nm for 1 min at 30 °C, pH 6.0. Data points are representative of three experiments. B, HPLC elution profile of products of MAPK peptide hydrolysis by STYX-G120C fusion protein. All data were taken at the times indicated, after incubation of G120C mutant (13.1 μM final concentration) with 1 mM diphosphorylated peptide (DHTG-FLpTEpYVATR) at 30 °C, pH 6.0. The same volume of each sample was loaded on a C18 column and resolved by reverse phase HPLC as described under "Experimental Procedures." Peaks of absorbance at 220 nm are labeled with the phosphorylation state of the peptide as determined by standards (8). Elution profiles are offset for clarity.

dsPTPase, VHR (8), including inhibition by vanadate, suggesting that the Gly-containing STYX possessed all the structural components necessary for phosphorylated substrate binding similar to dsPTPases. We realize that the artificial substrates, pNPP and diphosphorylated peptides, only serve to indicate the potential function of STYX. The search for an in vivo phospho-protein(s) which selectively bind to STYX is currently underway.

We believe that STYX is the first example of a naturally occurring binding domain that is structurally similar to dsPTPases. Recent enzymatic (8) and crystallographic studies have implicated aspartic acid and arginine residues, as well as the amide backbone of the conserved catalytic loop, in coordinating and binding substrate phosphoryl groups by dsPTPases. Since STYX contains these structural components of the binding pocket, it may share a degree of overlap in substrate preference with dsPTPases. If this is the case, the function of STYX may be to bind phosphorylated dsPTPase substrates and thereby protect them from serine/threonine phosphatases. The existence of a "protective factor" has been proposed recently for MAPK signaling (5), and STYX would be predicted to have properties consistent with this function. The biological properties of STYX are currently under investigation.

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