Association of the protein tyrosine phosphatase PTP1C with the protein tyrosine kinase c-Src in human platelets

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Abstract Protein tyrosine phosphatase 1C (PTP1C), highly expressed in hematopoietic cells, is a soluble protein tyrosine phosphatase containing two Src homology 2 (SH2) domains at the N-terminus and two putative sites of tyrosine phosphorylation at the C-terminus. This paper reports that PTP1C and c-Src could be coimmunoprecipitated during thrombin-induced platelet activation. Moreover, association between the two signalling proteins occurred only after PTP1C had been tyrosine phosphorylated. In in vitro experiments, PTP1C bound to the SH2 domain of c-Src, suggesting that association between tyrosine phosphorylated PTP1C and c-Src was mediated by the SH2 domain of c-Src. Finally, in resting platelets, PTP1C was mainly found in the Nonidet P-40 soluble fraction whereas following thrombin-induced activation, around 17% of PTP1C was associated with the insoluble fraction.

Key words: Protein tyrosine phosphatase 1C; c-Src; SH2 domain; Human platelet

1. Introduction

Numerous phosphotyrosine proteins have been detected in normal non-proliferative and terminally differentiated cells such as platelets [1]. Most tyrosine proteins are phosphorylated during platelet activation induced by physiological agonists such as thrombin and collagen [2–4], as well as by thapsigargin [5,6], or the monoclonal antibody P256 directed against the integrin $\alpha_{IIb}\beta_3$, the receptor for fibrinogen also named glycoprotein IIb-IIIa [7]. Among the tyrosine phosphorylated proteins described in platelets, some have been detected in cellular contact regions and are related to platelet aggregation such as the focal adhesion kinase FAK [8]. However, most of them have not yet been identified and their roles remain unknown.

Several protein tyrosine kinases (PTKs) have been identified in platelets. c-Src is highly expressed together with some others of the same family [2,3,9]. Other soluble PTKs have also been identified such as FAK, Syk, a tandem Src homology 2 (SH2) domains kinase [10] and Matk, a Csk-like protein [11]. Modulation of platelet tyrosine kinase activity depends among others on intracellular Ca^{2+} [6], on activation of protein kinase C, or on activation of the integrin $\alpha_{IIb}\beta_3$ [12].

Phosphorylation at tyrosine residues being the result of a balance between PTK and protein tyrosine phosphatase (PTP) activities, some PTPs have also been characterized in human platelets. One of these, PTP1C, also named SHPTP1, HCP and SHP [13–16], is expressed in many hematopoietic cells, and is notably present in platelets [14,17]. It possesses a large non-catalytic region at the N-terminus with two adjacent SH2 domains, suggesting that it may interact with phosphotyrosine proteins [18,19]. PTP1C was first described to be phosphorylated on tyrosine in macrophages in response to colony stimulating factor-1 [20]. Later, two putative sites of tyrosine phosphorylation were described at the C-terminus [21,22]. In platelets, it is not clearly established whether this phosphatase plays a role in an early signalling event or in an aggregation-dependent mechanism. Indeed, PTP1C phosphorylation occurs within the first seconds of stimulation [23,24]. However, depending on the reports, this phosphorylation follows platelet aggregation [23] or does not [24]. Moreover, translocation of c-Src and PTP1C to the platelet cytoskeleton has been described, but up to now there is no evidence of any interaction between the two molecules. The results presented here confirm that PTP1C was rapidly phosphorylated on tyrosine in human platelets after stimulation with thrombin, and show that this phosphorylation allows PTP1C to bind the SH2 domain of c-Src. The functional implication of this binding is discussed.

2. Materials and methods

2.1. Antibodies, materials and chemicals

Polyclonal R278 and monoclonal anti-PTP1C antibodies were from Dr. E.R. Stanley (Albert Einstein College of Medicine, New York, USA) and from Transduction Laboratories (Affiniti, Mamhead, UK), respectively. Polyclonal N-16 and monoclonal GD11 anti-Src antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and from UBI (Euromedex, Souffelweyersheim, France), respectively. Monoclonal anti-phosphotyrosine antibody 4G10 was from Dr. B.J. Druker (Oregon Health Science University, Portland, USA) [25]. The pGEX vectors which contain glutathione *S*-transferase (GST) or GST-Src SH2 domain fusion protein were obtained from Dr. L.C. Cantley (Harvard Medical School, Boston, USA), and GST-PTP1C SH2 domains, which associates GST with the two SH2 domains of PTP1C, from Dr. T. Yi (St Jude Children's Research Hospital, Memphis, USA).

All SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were from Bio-Rad (Irvy sur Seine, France), and Na_3VO_4 from Fisher Scientific (Fair Lawn, USA). All other chemicals were of the highest available purity.

2.2. Platelet preparation, activation and Western blotting

Blood from healthy donors was anticoagulated in 0.1 vol. of ACD-C. Platelets were isolated from plasma on a metrizamide gradient (Eurobio, Les Ulis, France), as previously described [26], resuspended in 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$,

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Abbreviations: PTK, protein tyrosine kinase; SH2 domain, Src homology 2 domain; PTP, protein tyrosine phosphatase; GST, glutathione *S*-transferase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; pTyr, phosphotyrosine; NP-40, Nonidet P-40.

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10 mM glucose and 10 mM HEPES, pH 7.4, and adjusted to 4×10^8 cells/ml. Stimulation was performed on 0.4 ml aliquots in the cuvette of an aggregometer at 37°C under constant stirring (1100 rpm) in the presence of 1 mM $CaCl_2$. Platelets were stimulated with 0.1 U/ml bovine thrombin (Hoffmann La Roche, Basel, Switzerland), and then solubilized in sample buffer containing 5% β-mercaptoethanol and boiled for 5 min. Proteins were separated by SDS-PAGE [27] before transfer to nitrocellulose membrane. Membranes were incubated in a blocking solution (100 mM NaCl, 20 mM Tris, pH 7.4) containing 5% low fat dry milk before probing with the specific antibody. Detection was performed with an enhanced chemiluminescence system (ECL, Amersham, Les Ulis, France). When a second probing was required, stripping was performed by washing in 62.5 mM Tris, pH 6.8, 0.1 M β-mercaptoethanol and 2% SDS at 60°C for 30 min. Membranes were washed, incubated in the blocking solution and probed with a second antibody.

2.3. Immunoprecipitation and binding to GST-fusion proteins

In an attempt to preserve associations between signalling proteins during solubilization, we used 1% Nonidet P-40 (NP-40). Platelets were lysed by incubation with 0.5 vol. of 3×lysis buffer (3% NP-40, 150 mM Tris-HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 1.5 mM Na_3VO_4, 150 mM benzamidine, 15 μg/ml leupeptin and 15 μg/ml aprotinin) for 30 min at 4°C. The insoluble fraction was sedimented by centrifugation at $16000\times g$ for 10 min. The soluble fraction was incubated with the polyclonal anti-PTP1C antibody R278 and immune complex precipitated with protein A-Sepharose.

Expression of GST fusion proteins was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and the fusion proteins were produced and purified from bacterial lysates as previously described [28]. The NP-40-soluble fraction of lysed platelets was incubated with fusion proteins bound to glutathione-Sepharose. In both cases, beads were solubilized in a SDS-PAGE sample buffer and proteins analyzed by Western blotting as described above.

2.4. ELISA

Plates were coated overnight with the polyclonal anti-PTP1C antibody R278 and incubated with a blocking solution containing 1% bovine serum albumin. Lysates and soluble fractions from 4×10^7 platelets were added to the wells and PTP1C probed with monoclonal antibody. *o*-Phenylenediamine was used as substrate and the absorbance read at 490 nm in a multichannel analyser (Dynatech MR 5000, Guyancourt, France). PTP1C was measured in platelet lysates and soluble fractions, and was expressed in percent of the absorbance in platelet lysates.

3. Results

3.1. Thrombin induces the tyrosine phosphorylation of PTP1C and its association with c-Src

Fig. 1A shows that stimulation of human platelets with thrombin induced and increased the tyrosine phosphorylation of several proteins, a result consistent with previous reports. Among them, a 64 kDa phosphotyrosine protein was identified as PTP1C by immunoprecipitation with anti-PTP1C antibody. In resting platelets PTP1C was not phosphorylated on tyrosine, nor was it associated with any phosphotyrosine protein. After stimulation, PTP1C was tyrosine phosphorylated. Tyrosine phosphorylation of PTP1C was also confirmed by immunoprecipitation with monoclonal antiphosphotyrosine antibody (data not shown).

After 3 min of activation, a 60 kDa phosphotyrosine protein coimmunoprecipitated with PTP1C. The 60 kDa phosphotyrosine protein was identified as c-Src by immunoblotting with a monoclonal anti-Src antibody (Fig. 1B). This association was observed after 30 s of activation and was more important after 1 min.

3.2. PTP1C binds in vitro the SH2 domain of c-Src

The interaction of PTP1C with c-Src was investigated using GST fusion proteins containing either c-Src or PTP1C SH2 domains (Fig. 2). In resting platelets the GST-Src SH2 domain associated with a 60 kDa phosphotyrosine protein and GST-PTP1C SH2 domains with one of 34 kDa.

After 3 min of thrombin-induced activation, the GST-Src SH2 domain associated with phosphotyrosine proteins of 42, 56, 60 (identified as c-Src [9]), 64 and 140 kDa. The 64 kDa protein was identified by specific immunoblotting as PTP1C. The association between PTP1C and the GST-Src SH2 domain was dependent on platelet activation since no association was observed in resting platelets. Under the same activating conditions, GST-PTP1C SH2 domains associated with unidentified phosphotyrosine proteins of 32, 34 and 38 kDa. A weak association of GST-PTP1C SH2 domains with c-Src was observed, which was not related to the high degree of c-Src tyrosine phosphorylation in the lysate.

3.3. Translocation of PTP1C to the NP-40 insoluble fraction

Following stimulation, many signalling proteins from platelets relocalize from a soluble fraction to an insoluble fraction. Fig. 3 shows that the amount of PTP1C in the insoluble fraction increased during thrombin-induced activation, suggesting a relocalization of PTP1C during thrombin-induced stimulation. Moreover, relocalization of PTP1C seemed to be slower than that of the main phosphotyrosine protein c-Src. In resting platelets c-Src was largely soluble and after 1 min of activation was present in insoluble fraction whereas PTP1C appeared only after 3 min. To quantify PTP1C relocalization, we developed an ELISA method and evaluated the amount of PTP1C present in soluble fraction (Table 1). By this method we estimated that 96% of total PTP1C was present in the soluble fraction of resting platelets compared to 83% present after 10 min of stimulation with thrombin. Thus, 17% of the total PTP1C was found in the actin cytoskeleton after 10 min of thrombin-induced activation.

4. Discussion

In the present report, we showed that PTP1C was phosphorylated on tyrosine after platelet activation [23,24], thereby forming a binding site to the c-Src SH2 domain.

Using immunoprecipitation of PTP1C and immunoblotting with an anti-phosphotyrosine antibody, we confirmed the increase in tyrosine phosphorylation of PTP1C during activa-

Table 1
Quantification of the percentage of PTP1C present in the NP-40 soluble fraction

	Time (min)				
	0	1	3	5	10
% of PTP1C in NP-40 soluble fraction	96 ± 3	93 ± 2	90 ± 9	83 ± 4	83 ± 1

Platelets were stimulated for the indicated times as described in Fig. 1. Quantification of PTP1C in soluble fractions of platelet lysates was performed by ELISA as described in section 2. Results are expressed in percent of the total amount and are mean ± S.E. of duplicate determinations in four experiments.

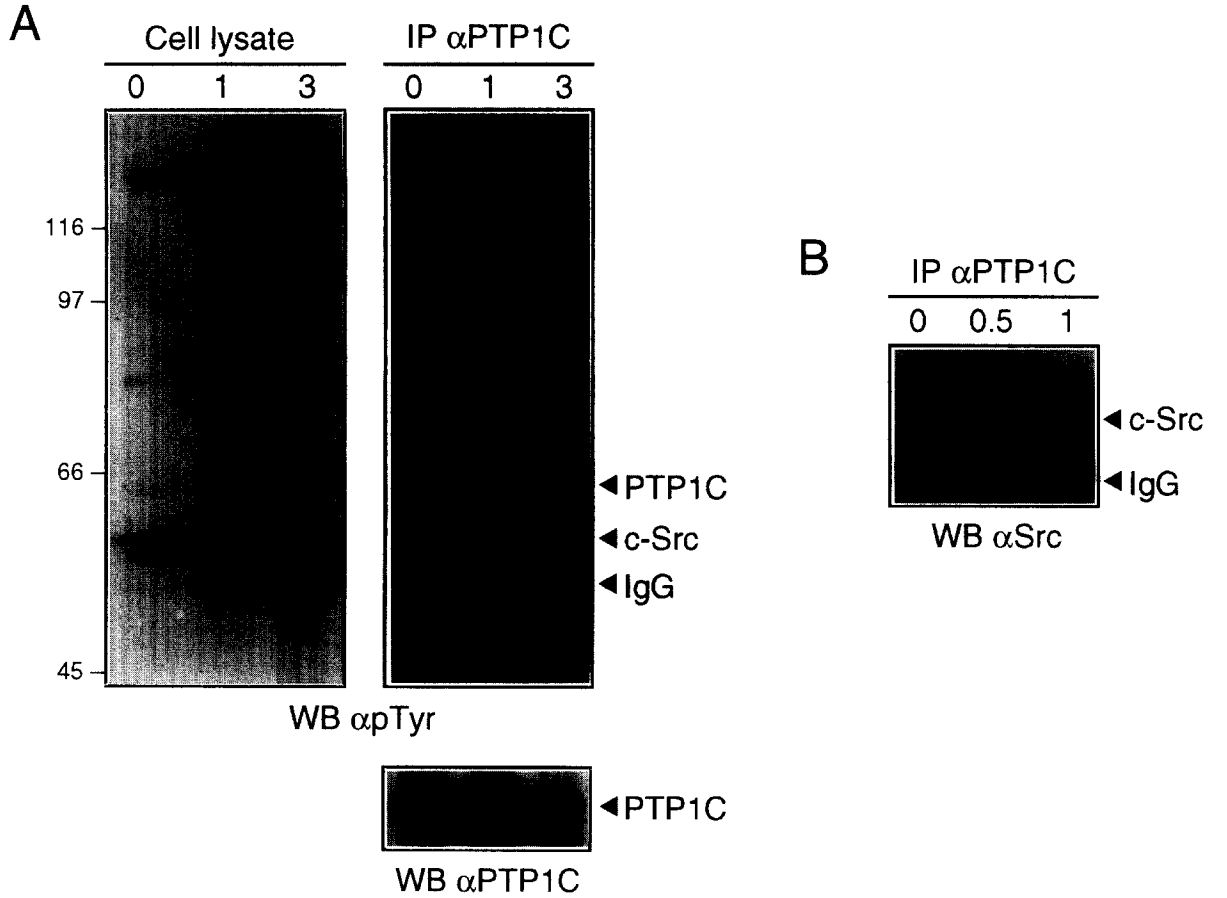

Fig. 1. Thombin induces the tyrosine phosphorylation of PTP1C and its association with c-Src. (A) Platelets were stimulated with 0.1 U/ml thrombin for 1 and 3 min at 37°C under constant stirring in the presence of 1 mM $CaCl_2$. (Left) Platelet proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and immunoblotted (WB) with monoclonal anti-phosphotyrosine (pTyr) antibody. (Right) Soluble fraction of platelet lysates were immunoprecipitated (IP) with polyclonal anti-PTP1C antibody. Nitrocellulose membrane was immunoblotted with monoclonal anti-phosphotyrosine antibody (upper panel), stripped, and immunoblotted with polyclonal anti-PTP1C antibody (lower panel). (B) Platelets were stimulated with 0.1 U/ml thrombin for 0.5 and 1 min at 37°C under constant stirring in the presence of 1 mM $CaCl_2$. Soluble fractions of platelet lysate were immunoprecipitated with polyclonal anti-PTP1C antibody. Nitrocellulose membrane was immunoblotted with monoclonal anti-Src antibody. The 55 kDa band is the IgG heavy chain from the antibody used for immunoprecipitation. The positions of molecular mass markers are indicated in kDa.

tion [23,24]. The migration in SDS-PAGE of PTP1C corresponded to the migration of the prominent tyrosine protein of 64 kDa [29]. No dephosphorylation of PTP1C was observed with thrombin, even after 10 min of activation (data not shown). This result contrasts with the transient phosphorylation of PTP1C induced by growth factors in A431 and 293 cells [22].

Regulation of signalling proteins by their phosphorylation is a general feature. Increase in phosphatase activity upon tyrosine phosphorylation has been reported for PTP1C in platelets [24] and for CD45 in T lymphocytes [30,31]. Tyrosine phosphorylation also allows interactions with SH2 domains [18,19] or phosphotyrosine interaction domains [32,33]. The sequences surrounding the two phosphorylation sites of PTP1C (Tyr-538 and Tyr-566) are Tyr-Gly-Asn-Ile and Tyr-Glu-Asn-Leu, respectively [22]. These two sequences present some homology with the consensus sequence for binding to the Src SH2 domain (pTyr-Glu-Glu/Asn/Tyr-Ile/Met/Leu) [34]. Therefore, we examined the possibility that PTP1C could bind to c-Src during platelet activation. In non-activated platelets, unphosphorylated PTP1C was not associated with phosphorylated c-Src. When platelets were stimulated by thrombin, a fraction of c-Src was coimmunoprecipitated with PTP1C. This association was detectable after 30 s of activation, at which time PTP1C was already phosphorylated on tyrosine. Through in vitro experiments, tyrosine phosphorylated PTP1C was shown to bind to the GST-Src SH2 domain. Together, these results suggest that the tyrosine phosphorylation of PTP1C allows its binding to c-Src via the SH2 domain of c-Src.

The PTK responsible for the tyrosine phosphorylation of PTP1C has yet to be identified. Tyr-566 is phosphorylated by Src in v-Src-transformed rat fibroblasts [35] and Tyr-564 of SHPTP1 (corresponding to Tyr-566 of PTP1C) by Lck, a Src family PTK, in murine T cells [36]. Beside Src, other soluble platelet PTKs of the Src family (Fyn, Lyn, Hck, Yes), or Matk, are also potential candidates. Indeed, Matk presents some similarity with Csk which phosphorylates CD45 in T lymphocytes [30]. Through in vitro experiments it was shown that tyrosine phosphorylation of CD45 increases its phosphatase activity and creates a binding site for Lck SH2 domain with a higher affinity than its own pTyr-505, leading to dephosphorylation of pTyr-505 and Lck activation. Similarly, tyrosine phosphorylated PTP1C might displace c-Src pTyr-527 from its SH2 domain, then dephosphorylate pTyr-527 and thus activate c-Src. Such an activation mechanism, by

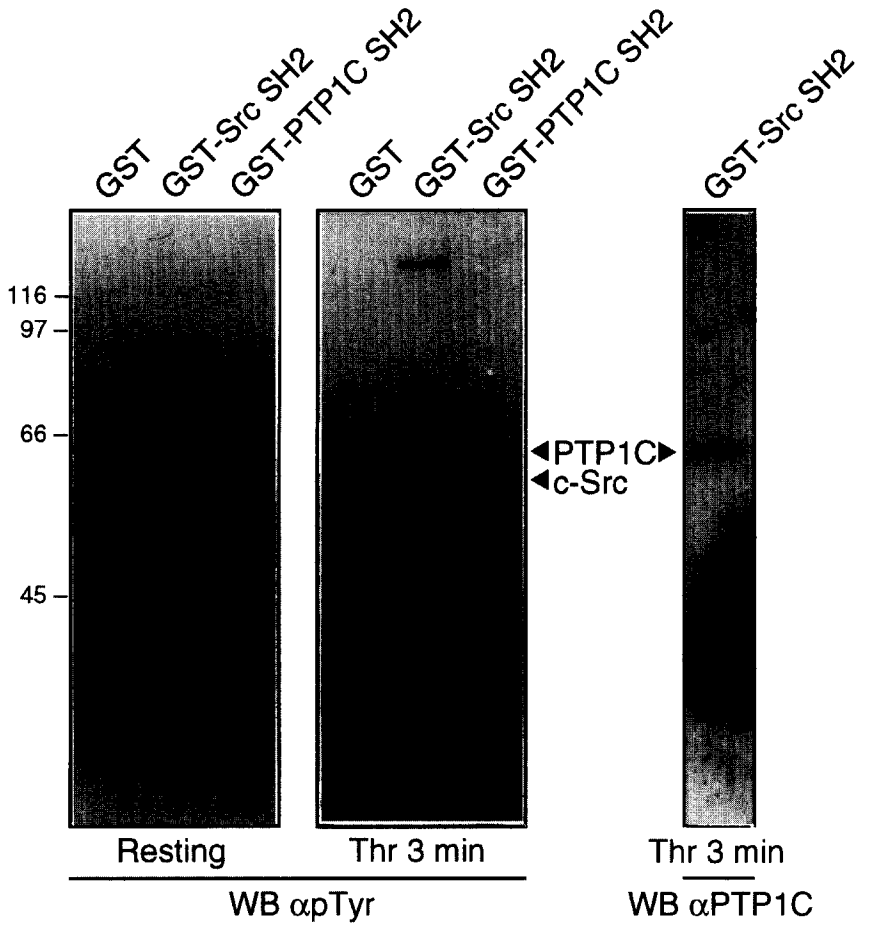

Fig. 2. The tyrosine phosphorylated PTP1C binds to GST-Src SH2 fusion protein. Platelets were not stimulated (Resting) or stimulated with thrombin for 3 min (Thr 3 min) as described in Fig. 1. Soluble fractions of platelet lysate were incubated with 2 μg GST fusion proteins immobilized on glutathione-Sepharose beads. Protein complexes were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with monoclonal anti-phosphotyrosine antibody (two left panels) or polyclonal anti-PTP1C antibody (right lane).

dephosphorylation of an inactive PTK, is now well documented. In Fischer rat embryo fibroblasts, overexpression of PTPα results in a persistent activation of c-Src by dephosphorylation at pTyr-527, with concomitant cell transformation and tumorigenesis [37]. Dephosphorylation of pTyr-527 of c-Src has also been observed in chicken preB DT40 cells overexpressing ChPTPλ [38]. In accordance with this hypothesis, c-Src pTyr-527 is rapidly dephosphorylated during thrombin-induced platelet activation [39].

Another feature of PTP1C concerns its translocation during activation. In control platelets, PTP1C was mainly found in the soluble fraction after NP-40 solubilization. Following thrombin activation, PTP1C was partially located in the insoluble fraction, as previously described [17]. The ELISA assay we have developed allowed an estimation of 4% of the total amount of PTP1C in the actin cytoskeleton of resting platelets, whereas 17% was found in this insoluble fraction after 10 min of thrombin-induced activation.

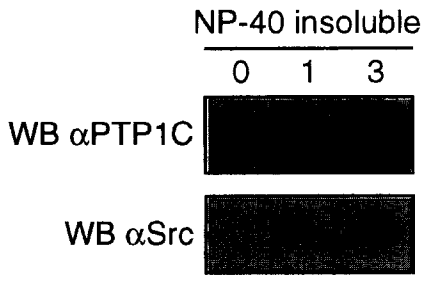

Fig. 3. PTP1C relocalizes in the NP-40 insoluble fraction during thrombin-induced activation. Platelets were stimulated for the indicated times as described in Fig. 1. Insoluble fractions were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with monoclonal anti-PTP1C (upper) or polyclonal anti-Src (lower) antibodies.

In conclusion, PTP1C appears to be implied both during initial times (early phosphorylation) and in later steps of platelet activation. Tyrosine phosphorylation, which increases PTP1C phosphatase activity [24], could allow its binding to the SH2 domain of c-Src. Thus, c-Src could be a potential substrate for PTP1C becoming activated by dephosphorylation of pTyr-527.

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