

Src homology 2 domains of protein tyrosine phosphatase are associated in vitro with both the insulin receptor and insulin receptor substrate-1 via different phosphotyrosine motifs

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Abstract

To clarify the role of protein tyrosine phosphatase containing Src homology 2 (SH2) regions on insulin signaling, we investigated the interactions among the insulin receptor, a pair of SH2 domains of SH-PTP2 coupled to glutathione-S-transferase (GST) and insulin receptor substrate-1 (IRS-1)-GST fusion proteins (amino-portion, IRS-1N; carboxyl portion, IRS-1C). GST-SH2 protein of SH-PTP2 bound to the wild type insulin receptor, but not to that with a carboxyl-terminal mutation (Y/F2). Furthermore, even though Y/F2 receptors were used, the SH2 protein was also co-immunoprecipitated with IRS-1C, but not with IRS-1N. These results indicate that SH2 domains of SH-PTP2 can directly associate with the Y¹³²²TXM motif on the carboxyl terminus of insulin receptors and also may bind to the carboxyl portion of IRS-1, possibly via the Y¹¹⁷²IDL motif in vitro.

Key words: Insulin receptor; Protein tyrosine phosphatase; Insulin receptor substrate-1; Src homology 2; Phosphotyrosine motif

1. Introduction

Protein tyrosine phosphorylation states are regulated by both protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTPase) and are thought to be one of the crucial steps for the regulation of cell growth, differentiation and metabolism [1,2]. Activation of growth factor receptors results in the autophosphorylation of tyrosine residues in the cytoplasmic regions of the receptors. Subsequent transmission of the ligand-induced signal is thought to be dependent on the recognition of the phosphorylated tyrosine (phosphotyrosine motifs) by distinctive domains, known as the SH2 (Src homology 2) region [3,4]. Insulin receptor autophosphorylation stimulates

receptor PTK, and the autophosphorylation induces the association of insulin receptors with PTPase containing SH2 regions [5], possibly via the motif on the carboxyl terminus (Y¹³²²THM) [6,7]. On the other hand, insulin receptor substrate-1 (IRS-1) contains multiple potential tyrosine phosphorylation sites, and tyrosine-phosphorylated IRS-1 binds to phosphatidylinositol (PtdIns) 3'-kinase and GRB2, suggesting that the IRS-1 employs a discrete SH2-docking protein [8–13].

Several types of novel non-transmembrane PTPases which contain a single phosphatase domain and two adjacent copies of SH2 regions at its amino terminus have been reported [14–21]. Thus, it is speculated that the activation of PTK is regulated by the PTPase via an SH2 domain-mediated interaction with tyrosine-phosphorylated receptors. Therefore, we investigated the interactions between a pair of SH2 domains of PTPases (SH-PTP2, PTP1C) and insulin receptors to clarify the role of PTPases containing the SH2 region on insulin signaling. We found that the GST-SH2 protein of SH-PTP2, but not that of PTP1C, may associate with the carboxyl-terminus of insulin receptors [5].

To clarify the significance of this PTPase in insulin signaling, we further identified not only a putative binding site of SH2 protein in the carboxyl terminus of insulin receptors, but also a possible binding site for IRS-1 with this SH-PTP2.

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Abbreviations: SH2, Src homology 2; IRS-1, insulin receptor substrate-1; IRS-1N, amino-portion of IRS-1 (amino acids 434–764); IRS-1C, carboxyl-portion of IRS-1 (amino acids 1132–1235); GST, glutathione-S-transferase; Y/F2, a mutant receptor in which both carboxyl-terminal tyrosine residues (1316 and 1322) are replaced by phenylalanine; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PtdIns 3'-kinase, phosphatidylinositol 3'-kinase; WGA, wheat germ agglutinin; HIRc cells, Rat 1 fibroblasts expressed with human insulin receptors; α IR, anti-insulin receptor antiserum; α GST, anti-GST antiserum; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor.

2. Materials and methods

2.1. Materials

Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo and Toyobo. Recombinant Taq polymerase was obtained from Perkin-Elmer Cetus. Oligonucleotide primers were synthesized on an Applied Biosystem 380A synthesizer. The vector pGEX-3X and wheat germ agglutinin (WGA) agarose were obtained from Pharmacia PL Biochemical Co. Purified porcine insulin was a gift from Novo-Nordisk Pharmar Ltd. ^{125}I -labeled porcine insulin at A14 (A14- ^{125}I insulin; 2200 Ci/mmol) was obtained from New England Nuclear. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Amersham International plc. Anti-insulin receptor antiserum (α IR) was obtained from a Type B insulin resistant patient with Sjögren's syndrome as previously described [22]. Rat 1 fibroblasts stably over-expressed human insulin receptors (HIRc) and the expression vector for carboxyl-terminal mutant insulin receptors (cvsvHIRY/F2) in which both carboxyl-terminal tyrosine residues (1316 and 1322) were replaced by phenylalanine, were previously described [23,24]. COS 7 cells and the transient expression vector for human insulin receptor (pGEM3SVHIR) were gifts from Dr. H. Teraoka (Shionogi Research Laboratory, Osaka, Japan) [25]. All other reagents were of analytical grade from Sigma, Bio-Rad and Nakarai Chemical Co.

2.2. Cell culture

Both HIRc and COS 7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

2.3. Polymerase chain reaction (PCR) and subcloning of the cDNA for IRS-1

Rat liver genomic DNA was isolated by the standard methods. PCR primers for IRS-1 were designed to amplify the regions that contained the 1st to 6th YXXM (YXXM) motifs at the amino terminus (amino acids 434–764, 993 bp from ref. [8], designated IRS-1N), and the carboxyl terminus (amino acids 1132–1235, 312 bp; IRS-1C). PCR proceeded in an air thermal cycler (Idaho model 1620) for 30 cycles (94°C for 10 s, 55°C for 20 s and 72°C for 40 s). The PCR products were subcloned into sequence vectors (pUC118,119) and the pGEX-3X expression vector with restriction enzyme (*Eco*RI and *Bam*HI) cleavage sequences attached to the PCR primers. Nucleotide sequences of subcloned DNA were determined using an automated laser fluorescent sequencer (Pharmacia).

2.4. Purification of GST fusion proteins

SH2 protein of SH-PTP2 coupled to GST was synthesized by PCR as previously described [5]. Bacterial GST fusion proteins were obtained by the recommended protocol. GST-SH2-SH-PTP2, GST-IRS-1N and GST-IRS-1C were purified using affinity column chromatography. GST-SH2 protein of SH-PTP2 was cleaved by factor X (Takara), then the SH2 protein of SH-PTP2 (23 kDa) was purified. An anti-rabbit GST polyclonal antiserum (α GST) was raised by the standard method, and this antibody immunoprecipitated more than 90% of the GST fusion proteins (GST-SH2-SH-PTP2, GST-IRS-1N and GST-IRS-1C) under our experimental conditions (data not shown).

2.5. Purification of insulin receptors and preparation of the carboxyl-terminal truncated and mutated insulin receptors

Insulin receptors were partially purified using WGA from HIRc cells and ^{125}I insulin binding to purified receptors was assessed using polyethylene glycol as previously described [23,26]. According to the method of Goren et al. [27], the 85-kDa carboxyl-terminal truncated insulin receptors were prepared by trypsin digestion (10 $\mu\text{g}/\text{ml}$ for 1 min at 22°C). The pGEM3SV-HIRY/F2 expression plasmid was constructed by replacing the *Bgl*III–*Spe*I fragment of the insulin receptor cDNA of pGEM3SVHIR with that of the cvsvHIRY/F2 plasmid. Carboxyl-terminal mutated (Y/F2) insulin receptors which lost the Y¹³²²TXM motif at the carboxyl terminus were obtained from COS 7 cells transfected with the pGEM3SV-HIRY/F2 plasmid using DEAE-dextran [25].

2.6. Phosphorylation of SH2 protein of PTPase by Insulin receptor kinase

After incubating insulin receptors with 167 nM insulin for 16 h, normal, carboxyl-terminal truncated and carboxyl-terminal mutated

(Y/F2) insulin receptors (500 fmol insulin binding capacity) were incubated with GST-SH2 protein (25 μg) of SH-PTP2 in the presence of 100 μM ATP ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 30 $\mu\text{Ci}/\text{tube}$) for 3 h at 4°C and these proteins were then immunoprecipitated by antibodies (α IR and α GST) and resolved by SDS-PAGE.

2.7. Interaction of SH2 protein of PTPase and GST-IRS-1 fusion proteins

We next examined the association of SH2 protein with phosphorylated GST-IRS-1 fusion proteins (GST-IRS-1N and GST-IRS-1C) as well as insulin receptors. After insulin stimulation, GST-IRS-1 fusion proteins (20 μg) were phosphorylated by either wild type or mutant insulin receptor (500 fmol insulin binding capacity) in the presence of 100 μM ATP ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 30 $\mu\text{Ci}/\text{tube}$) for 3 h at 4°C. GST-uncoupled SH2 protein (30 μg) of SH-PTP2 was added into this mixture containing receptor and IRS-1 and incubated for a further 3 h at 4°C. These proteins were immunoprecipitated with antibody (α GST) then resolved to SDS-PAGE.

3. Results

We studied whether the putative binding site for SH2 protein was Y¹³²²THM at the carboxyl terminus of insulin receptors. We found that wild-type insulin receptors phosphorylated the GST-SH2 protein of SH-PTP2 and were co-immunoprecipitated with this SH2 protein by GST antiserum. On the other hand, the carboxyl-terminal-truncated insulin receptors failed to associate with SH2 protein, suggesting that the SH2 domains of SH-PTP2 associate with the carboxyl terminus of insulin receptors. Furthermore, the carboxyl-terminal-mutated (Y/F2) insulin receptors, where this motif in the carboxyl terminus was destroyed by replacing tyrosine residues with phenylalanine, phosphorylated GST-SH2 protein, but failed to associate with the SH2 protein, as did the carboxyl-terminal truncated insulin receptors (Fig. 1). These results indicate that the SH2 protein of SH-PTP2 binds to Y¹³²²TXM motif at the carboxyl terminus of insulin receptors.

To assess whether this SH2 protein of SH-PTP2 bound to IRS-1, and if so, where the putative binding site of SH2 protein was located in IRS-1, we constructed GST-IRS-1 fusion proteins (GST-IRS-1N and GST-IRS-1C), and studied the association of these GST-IRS-1 proteins with GST-uncoupled SH2 protein of SH-PTP2. Phosphorylated SH2 protein co-immunoprecipitated with GST-IRS-1C, but GST-IRS-1N was not affected by anti-GST antibody, suggesting that the phosphorylated GST-IRS-1N failed to bind to SH2 protein of SH-PTP2, but GST-IRS-1C bound to SH2 protein in vitro. Insulin receptor kinase phosphorylated GST-IRS-1N to a lesser extent than GST-IRS-1C under our experimental conditions. Thus, we used the more phosphorylated GST-IRS-1N protein. However, there was no evident association of GST-IRS-1 with the SH2 protein (data not shown). It remained possible that SH2 protein co-immunoprecipitated with IRS-1 proteins via the interaction of the carboxyl terminus of the insulin receptor. To rule this out, we used the carboxyl-terminal-mutated (Y/F2) receptor

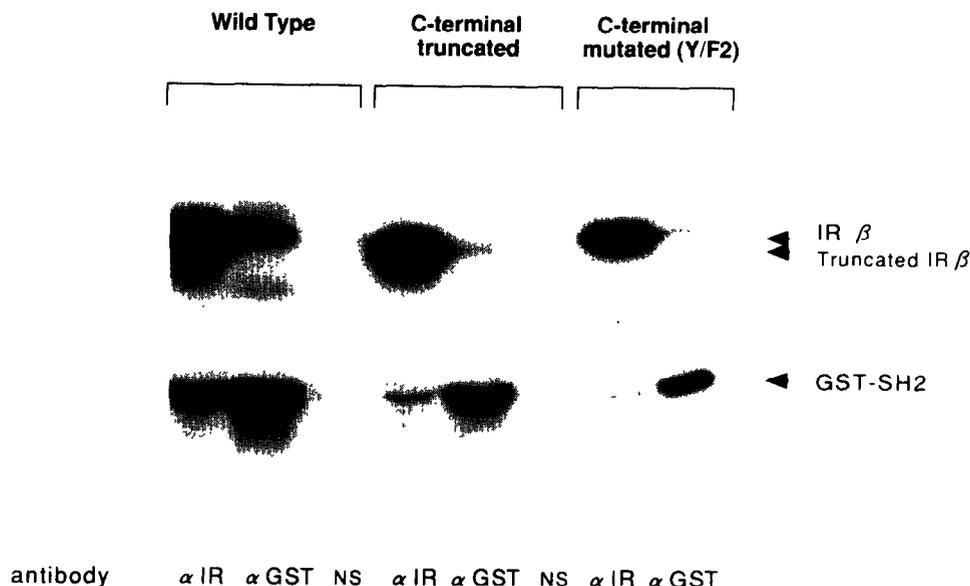


Fig. 1. Phosphorylation of the SH2 domains of PTPase by insulin receptor kinase and association of SH2 domains with the autophosphorylated carboxyl-terminus of the insulin receptor. After insulin stimulation, normal (wild-type), carboxyl-terminal-truncated, and carboxyl-terminal-mutated (Y/F2) insulin receptors were incubated with GST-SH2 fusion protein (50 kDa) in the presence of 100 μ M ATP at 4°C for 3 h, then these proteins were immunoprecipitated with anti-insulin receptor antiserum (α IR), anti-GST antiserum (α GST), and normal serum (NS), respectively and resolved by SDS-PAGE.

instead of the wild-type in these association studies. The SH2 protein was co-immunoprecipitated with GST-IRS-1C as shown in Fig. 2. These results suggest that the binding site for SH-PTP2 lies on the Y¹¹⁷²IDL motif on the carboxyl terminus of the IRS-1 molecule.

These findings suggest that the SH2 protein of SH-PTP2 can be phosphorylated by insulin receptor PTK and associate with insulin receptors via its Y¹³²²TXM motif on the carboxyl terminus of insulin receptors. However, several putative phosphotyrosine motifs (YMXM or YXXM) on the amino-terminus of IRS-1 cannot associate with SH2 domains. On the other hand, the YIDL motif on the carboxyl terminus of IRS-1 may bind to SH2 domains of this PTPase.

4. Discussion

Activated growth factor receptor PTK stimulates intracellular signaling pathways by binding to and phosphorylating regulatory cytoplasmic protein with phosphotyrosine motifs. It has been reported that PTPases homologous to SH-PTP2 can be phosphorylated in the cells stimulated by either platelet derived growth factor (PDGF) or by epidermal growth factor [20,21]. Furthermore, SH-PTP2 is phosphorylated at the tyrosine residues and directly binds to these growth factor receptors [28].

Even though the insulin receptor has a phosphotyrosine motif on the carboxyl-terminal (Y¹³²²THM) [6,7], there is no evidence that its autophosphorylation induces association with a signal protein containing SH2 regions.

However, we recently found that a pair of SH2 domains of SH-PTP2 are phosphorylated by insulin receptor PTK and that the insulin receptors are co-immunoprecipitated with GST-SH2 protein or SH-PTP2 by anti-GST antiserum, suggesting that SH2 domains of SH-PTP2 directly

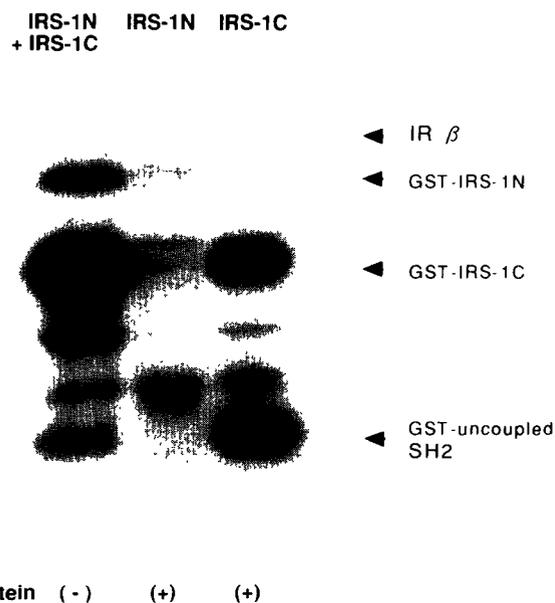


Fig. 2. Association of phosphorylated GST-IRS-1 fusion proteins with GST-uncoupled SH2 protein of SH-PTP2. After insulin stimulation, carboxyl-terminal-mutated (Y/F2) insulin receptors and GST-IRS-1 fusion proteins (GST-IRS-1N, and GST-IRS-1C, 20 μ g each) were incubated in the presence of 100 μ M ATP at 4°C for 3 h. GST-uncoupled SH2 protein (23 kDa) was then added to the mixture and incubated for a further 3 h. These proteins were immunoprecipitated with anti-GST antiserum (α GST), and resolved by SDS-PAGE.

associate with insulin receptors [5]. In this study, we also found that the carboxyl-terminal-truncated and carboxyl-terminal-mutated Y/F2 insulin receptors phosphorylated SH2 protein, but could not bind to it, indicating that the YTXM motif at 1322 on the carboxyl terminus of the insulin receptor is a binding site for the SH2 domains of SH-PTP2. Based upon the peptide binding analysis of growth factor receptors such as PDGF receptors, Y(I/V)X(V/I/L/P) motifs are putative binding sites for the N-terminal SH2 domain of SH-PTP2 [29]. However, the Y¹⁰⁰⁹TXV motif of the PDGF β receptor is the binding site of SH-PTP2 and it is speculated that the binding site of SH-PTP2 is a Y (hydrophobic/neutral amino acid) X V motif in vivo [30]. The phosphotyrosine motif at the carboxyl terminus of the insulin receptor is YTXM, which is similar to the YTXV motif in the PDGF receptor.

When the insulin receptor becomes activated, it phosphorylates IRS-1 on numerous tyrosine residues within seconds. IRS-1 may function as a docking protein, bringing various component proteins to the insulin signaling pathway [8–13]. For SH-PTP2, it is still unclear whether IRS-1 plays the role of a docking protein like PtdIns3'-kinase and GRB2. Syp, the mouse counterpart of SH-PTP2 co-immunoprecipitates with IRS-1 [31]. However, the binding site(s) of SH-PTP2 for IRS-1 remains unclear. According to a peptide binding study [29], the Y⁸⁹⁵VNI and Y¹¹⁷²IDL motifs may be the putative binding sites for SH-PTP2 in IRS-1. In this study, we found that the carboxyl-portion of IRS-1 that contained the Y¹¹⁷²IDL motif, bound to the SH2 protein. It is still unclear whether the Y⁸⁹⁵VNI motif is the binding site for SH-PTP2. Furthermore, IRS-1C also contains a putative tyrosine phosphorylation site at Y¹²²²ASI as well as at Y¹¹⁷²IDL. However, the Y¹²²²ASI motif is not consistent with the possible binding motifs as previously reported. To identify the exact binding motif for SH-PTP2 of the carboxyl-terminus of IRS-1, further studies using the IRS-1 mutants, where these possible binding motifs are destroyed, are now going to be investigated in our laboratory. On the other hand, the amino-portion of IRS-1 containing the 1st to 6th YXXM motifs failed to bind to SH2 protein. With regard to these differences in the phosphotyrosine motifs of various proteins for the binding of SH2 protein of SH-PTP2 (YIDL and YTXM), it has been suggested that the N-terminal SH2 domain of SH-PTP2 is least selective compared with other SH2 regions [29]. Therefore, the SH2 domains of SH-PTP2 may bind to the other motifs such as the YTXM in addition to the YIDL motif in the carboxyl portion of IRS-1. Alternatively, the tertiary structure of the binding site surrounding these motifs may be an important determinant of the binding affinity for SH2 domains of this PTPase as well as phosphotyrosine motifs of the amino acid residues.

SH-PTP2 mRNA is widely distributed throughout the

body, including insulin-sensitive tissues such as liver and skeletal muscles, and PTPases homologous to SH-PTP2 have little or no effect on the phosphorylation state of the insulin receptor PTK, although the expression of PTP1C leads to the dephosphorylation of receptor PTK including insulin receptors [21]. However, it is still unknown whether SH-PTP2 can dephosphorylate tyrosine phosphorylated IRS-1. In this study, we demonstrated that SH-PTP2 is phosphorylated by insulin receptor kinase and it is associated with the Y¹³²²TXM motif on the carboxyl terminus of the insulin receptor. Simultaneously, it may also bind to the Y¹¹⁷²IDL motif on the carboxyl terminus of IRS-1. This association of SH-PTP2 with either insulin receptor or IRS-1 may modulate a phosphorylation–dephosphorylation cascade of insulin action.

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