Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 208-214

Src homology 2 domains of Protein Tyrosine Phosphatase are phosphorylated by Insulin Receptor Kinase and bind to the COOH-terminus of Insulin Receptors *in vitro*

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Received May 24, 1993

Summary To clarify the role of protein tyrosine phosphatases(PTPase) containing Src homology 2 (SH2) regions on insulin signaling, we investigated the interactions between SH2 regions of PTPase and insulin receptors. We made a pair of SH2 domains of PTP1C and SH-PTP2 fusion proteins coupled to glutathione-S-transferase (GST) using pGEX-3X expression vector. After incubating with insulin, insulin receptors were incubated with SH2 proteins in the presence of 100 μ ATP at 4℃ for 3 hr, and then immunoprecipitated and analyzed by SDS-PAGE. We found that SH2 domains of SH-PTP2 were phosphorylated, but not those of PTP1C by insulin receptor kinase and the SH2 domains of SH-PTP2, but not those of PTP1C, directly bound to the phosphorylated COOH-terminus of insulin receptors *in vitro*.

Protein tyrosine phosphorylation is regulated by the activities of both protein tyrosine kinase(PTK) and protein tyrosine phosphatase(PTPase) and thought to be one of key steps that regulate cell growth, differentiation and metabolism(1,2). Activation of the transmembrane 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 containing approximately 100 amino acid residues, known as SH2 (Src homology 2) region(3,4).

Insulin receptor autophosphorylation stimulates receptor PTK, but there is as yet no evidence that this induces strong association with signal protein containing SH2 region, even though insulin receptor has such a motif on COOH-terminus(Y^{1322}THM) (5,6). However, insulin receptor substrate 1(IRS-1) contains multiple potential tyrosine phosphorylation sites (YMXM or YXXM motif), and tyrosine-phosphorylated IRS-1 binds and activates phosphatidylinositol (PtdIns)3'-kinase, suggesting that the insulin receptor employs a discrete

0006-291X/93 $4.00

SH2-docking protein, IRS-1, rather than incorporating its SH2 binding sites as an intrinsic part of the receptor(7-9).

Recently it has been reported novel non-transmembrane PTPase which contains a single phosphatase domain and two adjacent copies of SH2 regions at its amino terminus, suggesting that the activation of PTK may be regulated by SH2 domain-mediated interaction of tyrosine-phosphorylated receptor with PTPase. In these new family of PTPase containing a pair of SH2 domains, there are two subclasses, one predominantly expressed in hematopoietic cells(10-13), the other widely expressed in many tissues(14-20). Therefore, we have investigated the interactions between a pair of SH2 domains of two subclasses of PTPases(PTP1C and SH-PTP2) and insulin receptors to clarify the role of such PTPase containing SH2 region on insulin signaling.

Methods and Materials

Materials - Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo and Toyobo. Recombinant Therus aquatics(Taq) polymerase was obtained from Perkin-Elmer Cetus. Oligonucleotide primers were synthesized on Applied Biosystem 380A synthesizer. pGEX-3X vector and wheat germ agglutinin (WGA) agarose were obtained from Pharmacia PL Biochemical Co. Purified porcine insulin was a gift from Novo-Nordisk Pharmar Ltd. Pork insulin ^{125}I labeled at A14(A14-^{125}I-insulin; 2200Ci/mmol) and Bolton-Hunter reagent was obtained from New England Nuclear. [γ -^{32}P]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(21), and anti-phosphotyrosine antibody(α Py) was gifted by Dr. H.Fujio (Osaka University). All other reagents were of analytical grade from Sigma , Bio Rad and Nakarai Chemical Co.

Cell culture - Human lymphoblastoid cells (IM9) were cultured in suspension with RPMI-1640 medium with 10% fetal calf serum(FCS) and Rat 1 fibroblasts expressed with human insulin receptors(HIRc) kindly gifted by Dr. JM Olefsky, (University of California, San Diego) were maintained with Dulbecco modified essential medium with 10%FCS.

Polymerase chain reaction (PCR) and subcloning of SH2 domains of PTPase cDNA - Total RNA from IM-9 cells (5x 10^8 cells) was isolated by acid guanidium thiocyanate-phenol-chloroform extraction (AGPC) and converted to single stranded cDNA by oligo-dT priming and M-MLV reverse transcriptase(Superscript preamplification system, BML). These cDNAs were used as a template for *in vitro* amplification with Taq DNA polymerase (PCR). PCR primers for PTPase were design to amplify the pair of Src homology 2 (SH2) regions(amino acids 1-200, 614bp) of PTP1C(10) and those(amino acids 1-216, 648bp) of SH-PTP2 cDNA(15). The PCR was carried out with air thermal cycler(Idaho model 1620) for 30 cycles (92℃ for 15s, 54℃ for 20s and 72℃ for 30s). These PCR products were subcloned into the sequence vectors (pUC118,119) and pGEX-3X expression vector with restriction enzyme (*EcoRI* and *BamHI*) cleavage sequences attached to the PCR primers. Nucleotide sequences of subcloned DNA were determined with automated laser fluorescent sequencer(Pharmacia).

Purification of GST fusion proteins - Bacterial GST fusion proteins were obtained by recommended protocol. GST-SH2-PTP1C and GST-SH2-SH-PTP2 were purified with affinity column. Anti-rabbit GST polyclonal antiserum was obtained by standard method, and this antibody was able to immunoprecipitate more than 90% of GST fusion proteins containing SH2 domains (GST-SH2-PTP1C and GST-SH2-SH-PTP2) in our experimental conditions (data not shown).

Purification of insulin receptors and preparation of the COOH-truncated insulin receptors Insulin receptors were partially purified with WGA column from HIRc cells and ^{125}I-insulin binding to purified receptors was assessed by polyethylenglycol(PEG) method as previously described(22,23). Goren *et. al.*(24) reported that mild trypsin digestion of insulin receptors led to truncation of the COOH-terminus of receptors but preserving receptor PTK activity and we also conformed their findings using the mutant COOH-truncated insulin receptors(25). According to their method, the 85-kDa COOH-truncated insulin receptors were prepared by trypsin digestion (10 μ g/ml for 1min at 22℃).

Phosphorylation of SH2 domains of PTPase by insulin receptor kinase- After incubating with 167nM insulin for overnight, normal and COOH-truncated insulin receptors (500 fmol insulin binding capacity) were incubated with SH2 domains(25 μ g) of PTPases in the presence of 100 μ M ATP (γ -^{32}P-ATP, 30 μ Ci/tube) for 3 hr at 4℃ and then immunoprecipitated by antibodies(α IR, α Py, and α GST) and subjected to SDS-PAGE. Phosphorylated insulin receptors were separated from free ATP by Bio-Spin 30 column (Bio-Rad) and were incubated with ^{125}I-SH2 proteins labeled with Bolton-Hunter reagent and then immunoprecipitated and analyzed by SDS-PAGE.

Results

In order to clarify the role of the PTPase containing SH2 regions on insulin signaling, we investigated the interactions between insulin receptors and a pair of SH2 domains of two PTPases(PTP1C and SH-PTP2), respectively. We found that insulin receptor kinase phosphorylated SH2 domains of the SH-PTP2, but not those of PTP1C *in vitro* as shown in Figure 1. Some portion of the phosphorylation of SH2 proteins of SH-PTP2 was resistant to KOH treatment (1M KOH; 55℃ for 2hr). However, these phosphorylated SH2 proteins were weakly immunoprecipitated by α Py, suggesting that serine and threonine residues of SH2 domains might be mainly phosphorylated and a few portion of tyrosine residues might be phosphorylated by insulin stimulation.

Phosphorylated insulin receptors were interestingly co-immunoprecipitated with phosphorylated GST-SH2 domain of SH-PTP2 by anti-GST antiserum. These associations of phosphorylated insulin receptors and SH2 domains of SH-PTP2 were also seen even when SH2 domains were not phosphorylated as shown in Figure 2. Furthermore, we found that the COOH-truncated insulin receptors prepared by trypsin digestion were able to phosphorylate SH2 domains of SH-PTP2, but not able to bind to these SH2 proteins. On the other hand, the insulin receptors were not co-immunoprecipitated by α GST in the case of PTP1C. Furthermore, ^{125}I-labeled SH2 domains of PTP1C also failed to bind to insulin receptors (data not shown).

These results suggest that SH2 proteins of SH-PTP2, but not those of PTP1C are phosphorylated by insulin receptor PTK and the only SH2 domains of SH-PTP2 are associated with insulin receptors and the binding site for SH2 domains may lie on the COOH-terminus of insulin receptors, and the COOH-terminus of receptors may not be necessary for phosphorylation of SH2 domains by receptor PTK.

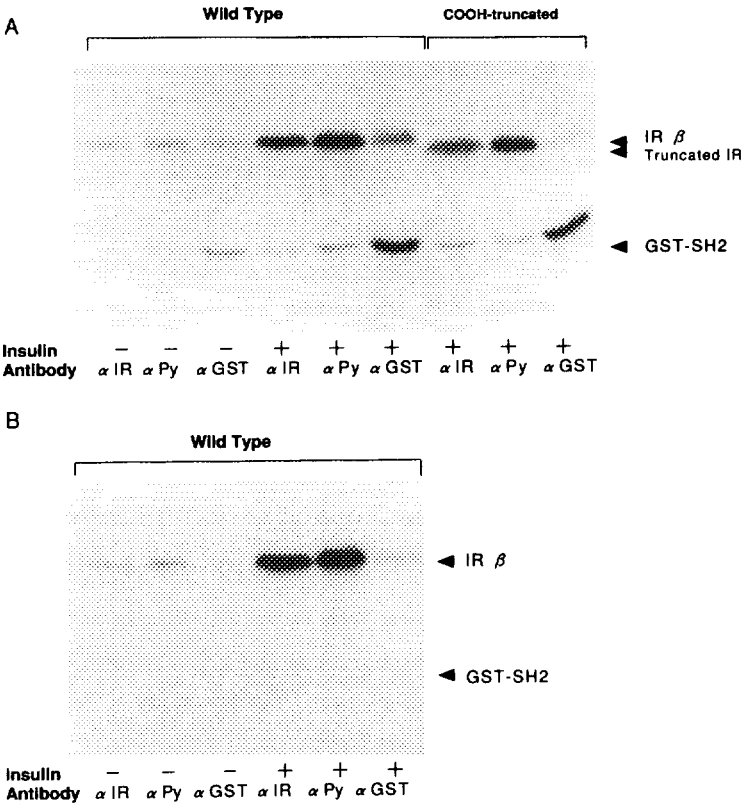

Figure 1.

Phosphorylation of SH2 domains of PTPases by insulin receptor kinase and binding of SH2 domains to autophosphorylated insulin receptors.

Either normal or COOH-truncated insulin receptor was incubated with SH2 fusion proteins in the presence of ATP at 4℃ for 3hr, and then immunoprecipitated by anti-insulin receptor antiserum (α IR), anti-phosphotyrosine antiserum (α Py) and anti-GST antiserum (α GST), respectively and analyzed by SDS-PAGE.

A. GST-SH2 domains of SH-PTP2; B. GST-SH2 domains of PTP1C.

Discussion

Activated receptor PTK stimulates intracellular signaling pathways by binding and phosphorylating regulatory cytoplasmic protein with phosphotyrosine motifs. Even though insulin receptor has such a motif on the COOH-terminus (Y^{1322}THM) (5,6), there is no evidence that insulin receptor autophosphorylation induces association with signal protein containing SH2 domains.

In the current study, we found that SH2 domains of SH-PTP2 were phosphorylated by insulin receptor PTK, whereas those of PTP1C were not phosphorylated. With regard to phosphorylation sites of SH2 proteins of SH-PTP2 by insulin receptor PTK, serine and

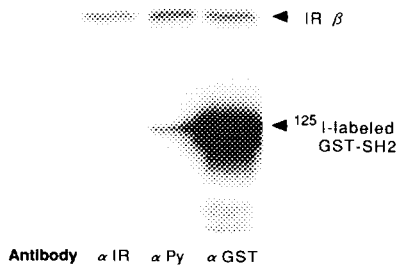

Figure 2.

Association of phosphorylated insulin receptors with unphosphorylated ^{125}I-labeled GST-SH2 proteins of SH-PTP2.

^{125}I-GST-SH2 proteins of SH-PTP2 labeled by Bolton-Hunter reagent and autophosphorylated insulin receptors were incubated in the absence of free ATP for 3hr at 4℃, and then immunoprecipitated, and analyzed by SDS-PAGE.

threonine residues might be mainly phosphorylated and a few portion of tyrosine residues might be phosphorylated by insulin stimulation. Further study is needed to clarify the phosphorylation sites of SH2 domains of SH-PTP2.

We also demonstrated that the insulin receptors were co-immunoprecipitated with GST-SH2 proteins of SH-PTP2 by anti-GST antiserum, suggesting that SH2 domains of SH-PTP2 were directly associated with insulin receptors, and the phosphorylation of SH2 domains were not necessary for these associations. On the other hand, SH2 domains of PTP1C were not able to bind to insulin receptors. Furthermore, it was interesting that the COOH-truncated receptors phosphorylated SH2 proteins but were not able to bind to SH2 proteins, suggesting that Y^{1334}XXM motif on the COOH-terminus of insulin receptor might be a SH2 domain binding site. This is the first evidence that insulin receptor autophosphorylation induces association with signal protein containing SH2 regions.

When SH2 proteins of SH-PTP2 were incubated with insulin receptors, insulin receptors were co-immunoprecipitated by α GST. However, GST-SH2 proteins were not co-immunoprecipitated by α IR. With respect to this discrepancy, it may be possible that only small percentage of SH2 proteins bind to insulin receptors. The other possibility such as a weak association of these complexes resulting in the easy dissociation of SH2 proteins from insulin receptors during the immunoprecipitation can be speculated. The exact explanation for this discrepancy is still unknown. However, the biological significance of the specific association of SH2 domains of SH-PTP2 to insulin receptors regarding to the insulin signaling mechanism awaits the further study.

When the insulin receptor becomes activated, within seconds it phosphorylates IRS-1 on numerous tyrosine residues. IRS-1 may function as docking protein, bringing various

component protein of insulin signaling pathway(7-9). In the case of SH-PTP2, it is still unclear whether IRS-1 may play the role of docking protein such as in the case of PtdIns3'-kinase.

Recently, it has been reported that PTP1C is phosphorylated at tyrosine residues by colony stimulating factor 1(CSF-1)(26) resulting that its PTPase activity is modulated(27). Furthermore, PTPases homologous to SH-PTP2 are phosphorylated in the cells stimulated by platelet derived growth factor(PDGF) and epidermal growth factor(EGF) and are associated with these receptors(19, 20). With regard to the differences between PTP1C and SH-PTP2, there are several points(10,15). First, there is striking sequence similarity between the SH2-containing PTPases, and they are much less similar to any other SH2-containing proteins(<40%), however, the SH2 domains of SHPTP2 are more similar to Drosophila corkscrew(76%) than to PTP1C(52-63%). Secondly, SH-PTP2 mRNA are widely distributed in many tissues including insulin-sensitive tissues such as liver, and skeletal muscles. On the other hand, PTP1C mRNA appears to be expressed predominantly in hematopoietic cell types. Finally, it has been reported that PTPases homologous to SH-PTP2 have a little or no effect on the phosphorylation state of the insulin receptor PTK(19, 28), although the expression of PTP1C leads to dephosphorylation of receptor PTK including insulin receptors(20,28).

Our findings in current study indicates that the SH-PTP2 may be phosphorylated by insulin receptor kinase and associates with the COOH-terminus of insulin receptor. In contrast with PTP1C which dephosphorylates autophosphorylated insulin receptor, SH-PTP2 may modulate insulin signaling by means of dephosphorylation of intracellular substrates of insulin receptor PTK. Alternatively undefined insulin receptor substrates, which are not PTPase but have SH2 regions highly homologous to those of SH-PTP2, may modulate insulin signaling. Further investigation will be necessary to evaluate roles of these enzymes in insulin action.

Acknowledgments

This work was supported by a grant-in-aid for encouragement of young scientists from the Ministry of Education, Science, and Culture, Japan.

We are grateful to Dr. JM.Olefsky(University of California, San Diego) for the gift of HIRc cells, and Dr. H. Fujio (Osaka University) for the gift of anti-phosphotyrosine antiserum.

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