Endocrine Journal 2000, **47** (4), 373–381

REVIEW

The Functional Significance of Shc in Insulin Signaling as a Substrate of the Insulin Receptor

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Introduction

THE activated insulin receptor phosphorylates various cellular substrates on tyrosine residues [1]. These substrates include the insulin receptor substrate family (IRS-1, -2, -3, -4). IRS functions as a multisite docking protein that interacts with the src homology 2 (SH2) domains of various signal-transducing molecules, including p85 subunit of PI3-kinase, Grb2, SHP2, Nck, and Fyn to propagate the insulin signal downstream [1]. Among them, PI3-kinase is a key molecule to mediate many of insulin-induced metabolic signaling [1]. Shc is another substrate of the insulin receptor [1, 2]. It is so named because Shc contains a SH2 domain and a Collagen-homologous region, and is composed of an amino-terminal phosphotyrosine binding (PTB) domain, a central collagen homology (CH) domain, and a carboxyl-terminal SH2 domain (Fig. 1) [3]. Anti-Shc antibody recognizes three distinct 46-, 52-, and 66-kDa isoforms of Shc [3]. All of these Shc isoforms originate from alternative splicing of a primary Shc transcript [3, 4]. The 46- and 52-kDa proteins are ubiquitously expressed except in the central nervous system, while the 66-kDa Shc protein is absent in some hematopoietic cells [3, 4]. These three classical Shc isoforms are also referred to collectively as ShcA [5, 6]. Two ShcA related genes, ShcB/Sck and ShcC/N-Shc, have been identified in the mouse and human [5, 6]. ShcB and ShcC are predominantly expressed in the brain, and may mediate the signaling from tyrosine kinases in the nervous system [5, 6]. In this review, we mainly describe the role of 46-, 52-, and 66-kDa Shc (ShcA) in insulin signaling. To facilitate the understanding of the Shc function, the specific roles of various Shc domains and tyrosine phosphorylation sites have been characterized. Furthermore, the role of Shc in insulin signaling has been compared with that in EGF signaling, because EGF functions only as mitogenic factor, whereas the physiological role of insulin is mainly metabolic.

This review was written as a memorial article for the Research Encouragement Prize of the Japan Endocrine Society awarded to Dr. Toshiyasu SASAOKA on June 5, 1998.
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Tyrosine phosphorylation sites of Shc

Shc becomes phosphorylated on tyrosine residues in response to various stimuli [2]. EGF receptor can similarly tyrosine phosphorylate all of the 66-, 52-, and 46-kDa Shc isoforms, whereas insulin receptor preferentially phosphorylates the 52-kDa, and to a lesser extent, the 46-kDa Shc isoforms [2]. An original study has indicated a Shc Tyr-317 residue as the critical phosphorylation site upon stimulation of growth factor receptors [3]. Thereafter, Shc Tyr-239 and Tyr-240 residues were identified as alternative phosphorylation sites [7–9]. Thus, Shc can be phosphorylated on Tyr-239/240 and/or Tyr-317 residues, which serve as docking sites for Grb2. EGF-induced activation of the Ras/MAP kinase pathway appears to be mainly dependent on the phosphorylation of Shc Tyr-317 in NIH-3T3 cells [9]. However, recent studies have also identified the importance of phosphorylation on Tyr-239/240

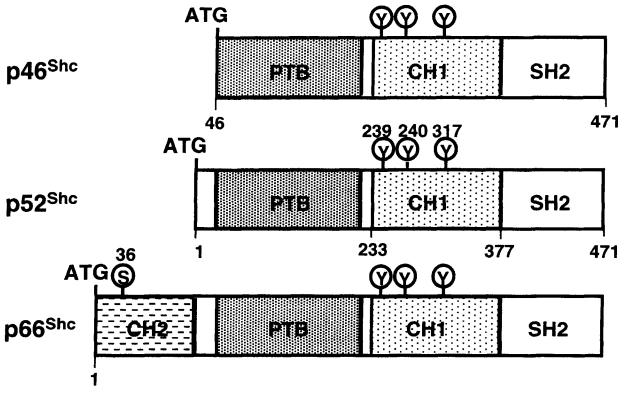

Fig. 1. Schematic structures of Shc isoforms. Structures of three Shc splicing isoforms are shown. $p46^{Shc}$ and $p52^{Shc}$ are composed of a phosphotyrosine binding (PTB) domain, a collagen homology 1 (CH1) domain, and Src homology 2 (SH2) domain, while $p66^{Shc}$ has an additional CH2 domain at the amino-terminus. S: a major serine-phosphorylation site of $p66^{Shc}$; Y: major tyrosine phosphorylation sites.

residues for the MAP kinase activation [8]. Interestingly, Drosophila Shc has tyrosine residues corresponding to Tyr-239/240 residues, but not Tyr-317 residue of mammalian Shc [10]. The fact that Shc Tyr-239/240 residues are highly conserved in evolution suggests that the phosphorylation of these residues is of fundamental importance. However, tyrosine-phosphorylated Drosophila Shc does not interact with Drosophila Grb2 in flies expressing the activated Drosophila EGF receptor homologue (DER) [10]. These results indicate that the signaling pathway from Shc to Grb2 occurred in evolution mainly via Shc Tyr-317 residue rather than Tyr-239/240 residues, although Shc also develops to utilize its Tyr-239/240 residues in a minor capacity to associate with Grb2 during the evolutional process, at least, in EGF signaling. Thus, the presence of two Grb2 binding sites in mammalian Shc protein adds to the complexity of Shc function. To clarify the role of Shc Tyr-239/240 and Tyr-317 residues in insulin signaling, we generated Shc cDNA with Tyr-317 → Phe (1F), Tyr-239/240 → Phe (2F), and Tyr-239/240/317 → Phe (3F) mutations [11, 12]. These mutant Shc plasmids were stably transfected into Rat1 fibroblasts overexpressing insulin receptors (HIRc), and insulin signaling was compared among these cell lines. Insulin-induced tyrosine phosphorylation of Shc, Shc·Grb2 association, and MAP kinase activation were significantly decreased in 1F-Shc and 3F-Shc cells, while these events were only slightly affected by expression of 2F-Shc [11, 12]. These findings indicate that Shc Tyr-317 residue is the predominantly insulin-induced phosphorylation site compared to Tyr-239/240 for coupling with Grb2, leading to MAP kinase activation in Rat1 fibroblasts (Fig. 2).

Shc interaction with the insulin receptor

The SH2 domain of Shc was originally shown to be a site to bind to the phosphorylated EGF receptor [3]. In this interaction, Shc appears to interact with the amino acid residues amino-terminal to phosphotyrosine of the receptor. This interaction is uncommon

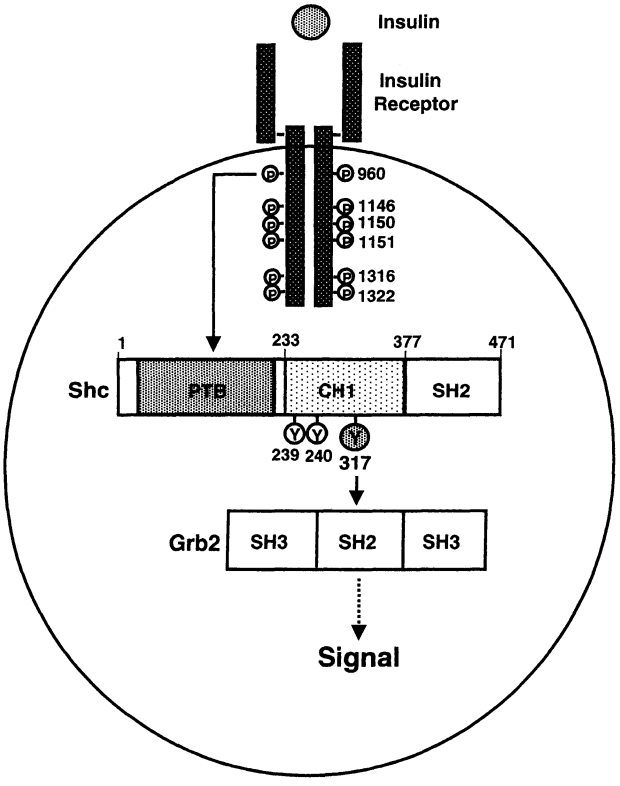

Fig. 2. Mechanisms of insulin-mediated Shc interaction with insulin receptor and Grb2. Upon insulin stimulation, the activated insulin receptor, via phosphorylated Tyr-960 residue, interacts with the PTB domain of Shc. Shc is then predominantly phosphorylated on 317-tyrosine residue among the three possible tyrosine phosphorylation sites for binding to the SH2 domain of Grb2.

for SH2 domains that usually interact with the amino acid residues carboxyl-terminal to phosphotyrosine [3]. In addition, the SH2 domain of Shc does not interact with the insulin receptor [13, 14]. Thereafter, the phosphotyrosine-binding (PTB) domain, that is distinct from that of all members of the known SH2 domain family, was shown to be capable of binding to phosphotyrosine residues [13–15]. Although tyrosine phosphorylated EGF receptors associate with both the SH2 domain and PTB domain of Shc, the activated insulin receptor interacts with Shc only via the PTB domain (Fig. 2) [13]. This interaction via Shc PTB domain is functionally important, because disruption of insulin receptor-Shc association by cellular microinjection of Shc-PTB GST fusion protein, but not Shc-SH2 GST fusion protein, effectively inhibited insulin-induced BrdU incorporation [13]. These results are consistent with the notion obtained by using yeast two-hybrid system, demonstrating insulin receptor interaction with Shc via the PTB domain [14, 15]. Phosphorylation of Tyr-960 residue of the juxta-membrane region of the insulin receptor β-subunit is required for the interaction with Shc [14, 15]. Because the IRS family, via the PTB domain, can also interact with the juxtamembrane domain around Tyr-960 of the insulin receptor, Shc and IRS can be considered as competitive substrates of the insulin receptor [11, 13].

The role of p46/52-Shc in insulin-induced mitogenesis

p21ras is a key molecule in the mitogenic signaling initiated by receptor tyrosine kinases including the insulin receptor [2, 16]. Since Grb2, an adaptor protein for the p21ras guanine nucleotide exchange factor Sos, can associate with IRS-1 independent of Shc, it is possible that insulin receptors can stimulate p21ras by interaction of IRS-1 with Grb2·Sos and that Shc is unnecessary for the signaling pathway [2, 16]. However, we demonstrated that Shc association with Grb2·Sos represents a predominant mechanism whereby insulin activates p21ras-MAP kinase [16]. Firstly, a larger amount of Grb2 was associated with Shc than with IRS [16]. Secondly, a significant amount of Sos activity for p21ras activation was seen in the anti-Shc immunoprecipitates, whereas only a negligible amount of Sos activity was associated with anti-IRS-1 immunoprecipitates in Rat1 fibroblasts expressing insulin receptors [16]. Based on these findings, the role of Shc in insulin-induced mitogenesis was proposed. To specifically identify the functional role of Shc in insulin-induced mitogenesis, we conducted single cell microinjection studies with anti-Shc antibody. The results demonstrated that microinjection of anti-Shc antibody into Rat1 fibroblasts expressing insulin receptors led to an 80% inhibition of subsequent insulin-induced DNA synthesis [2]. These findings indicate the importance of Shc in the mitogenic signaling of insulin. The role of Shc was further strengthened by gain-of-function experiments [11, 12]. Overexpression of wild-type $p52^{Shc}$ enhanced insulin stimulation of thymidine incorporation and BrdU incorporation. In contrast, expression of 1F- and 3F-$p52^{Shc}$, which are dominant negative forms of Shc by inhibiting the endogenous Shc function, led to a decrease in insulin-induced DNA synthesis [11, 12]. Taken together, Shc appears to play an important role in insulin induced mitogenesis via p21ras activation.

Regulation of p21ras activity by insulin

p21ras becomes active as a signaling molecule when it is converted from the GDP- to GTP-bound form, and this process is mainly controlled by Sos guanine nucleotide exchange factor for p21ras [2, 16]. The proline rich region of Sos binds to the SH3 domain of Grb2, and preformed Grb2·Sos complexes exist within unstimulated cells [2, 16]. Shc stimulates p21ras-GTP formation by interacting with the Grb2·Sos complex [16, 17]. By Shc binding to Grb2·Sos, Sos translocates from cytosol to transmembrane, where activation of p21ras occurs [19, 20]. In addition, insulin induced formation of the Shc·Grb2·Sos complex appears to increase the guanine nucleotide releasing activity of Sos [19]. However, prolonged p21ras activation, either by introducing oncogenic forms of p21ras or by activation of endogenous p21ras by the introduction of upstream activators such as membrane-targeted Sos, is a transforming event that plays a significant role in many malignancies [21]. The pattern of p21ras activation after insulin stimulation is a rapid increase in GTP-bound form of p21ras, peaking in the first several minutes and then falling rather rapidly

toward basal levels despite ongoing insulin stimulation [21]. Thus, strict control of p21ras activation is important in growth regulation of insulin [21]. In this regard, after insulin stimulation, Sos undergoes serine/threonine phosphorylation by MEK (MAP kinase kinase) dependent pathway [21, 22]. Phosphorylation of Sos causes dissociation of Sos from Grb2, while the Shc·Grb2 complex formation is still intact [21, 22]. Thus, dissociation of Sos from Grb2 plays a key role in limiting the duration of p21ras activation in response to ongoing insulin stimulation [21, 22]. By these mechanisms, insulin typically causes a transient rise in p21ras activity and the subsequent fall back to basal values to attenuate the insulin signaling (Fig. 3).

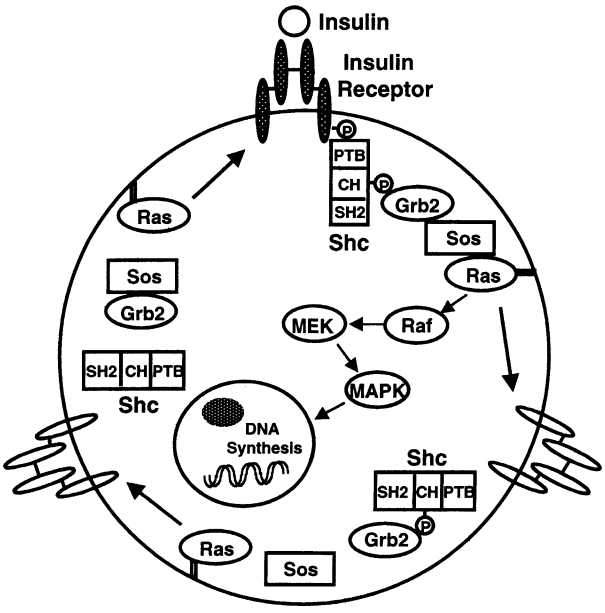

Fig. 3. Mechanisms of insulin mediated activation and inactivation of p21ras. In quiescent state, Shc and preformed Grb2·Sos complex reside separately in cytosol without interacting insulin receptors. Upon insulin stimulation, insulin receptor interaction with Shc results in Shc·Grb2·Sos formation. By forming the Shc·Grb2·Sos complex, Sos translocates from cytosol to the proximity of p21ras which localizes at the plasma membrane. The activated p21ras transmits signal to activate MAP kinase cascade leading to DNA synthesis. Thereafter, Sos is serine phosphorylated by MEK dependent pathway. The serine phosphorylated Sos dissociates from Grb2, while the Shc·Grb2 complex formation still remains, leading to p21ras inactivation.

The role of Shc in metabolic signaling

Insulin is a predominant hormone influencing the regulation of glucose metabolism [1]. One major effect of insulin is to augment glucose disposal by stimulation of glucose uptake into insulin's target tissues [1]. Skeletal muscle accounts for the majority of insulin-induced glucose disposal, and adipose tissue plays a certain role in insulin action [1]. However, experimental difficulties exist to analyze the role of Shc in insulin's target tissues. A sufficient amount of insulin receptors adequate for the signaling analysis is expressed only after cell differentiation [1]. In addition, studies with general transfection techniques have difficulties to express enough amount of exogenous Shc into differentiated skeletal muscle and fat cells. For these reasons, we employed a conventional way to examine the role of Shc by employing IGF-1 as an analogous hormone in L6 myoblasts [23, 24]. By utilizing transient expression procedure with lipofectamine, overexpression of WT-Shc increased IGF-1 induced Shc association with Grb2 and MAP kinase activity, resulting in enhanced DNA synthesis in L6 cells [24]. In addition, overexpression of Shc decreased IGF-1 induced tyrosine phosphorylation of IRS because of competitive interaction as substrates of the IGF-1 receptor [24]. Since these results are consistent with the previous findings seen with insulin in Rat1 fibroblasts [11, 13], IGF-1 studies in L6 cells appear to be a useful model to analyze the impact of Shc in metabolic signaling. Interestingly, overexpression of Shc inhibited downstream signaling of IRS [24]. Thus, IGF-1 stimulation of IRS-1 association with p85 subunit of PI3-kinase, PI3-kinase activity, Akt activity, and glycogen synthesis were all reduced by overexpression of Shc [24]. Furthermore, expression of Shc-PTB domain alone also inhibited IGF-1-induced tyrosine phosphorylation of IRS-1, Akt activity, and glycogen synthesis [24]. Taken together, Shc appears to inhibit IGF-1 induced metabolic signaling through inhibition of IRS-mediated IGF-1 signaling. Although the findings seen with IGF-1 may not completely apply to the signaling system in insulin's metabolic action, because of the similarities of signaling system between insulin and IGF-1, Shc may not be required for acute regulation of the metabolic actions of insulin.

The molecules that interact with Shc

Shc associates with a number of membrane receptor tyrosine kinases and non-membranous intracellular tyrosine kinases such as Src [3, 4]. Furthermore, Shc has been reported to associate with several other downstream signaling molecules in addition to Grb2 (Fig. 4) [25–30]. PEST protein tyrosine phosphatase is one such molecule and is the ubiquitously expressed cytosolic protein tyrosine phosphatase (PTPase) that is characterized by the presence of Pro, Glu, Ser, and Thr-rich PEST domains within the carboxyl terminus [25, 26]. Tyrosine phosphorylation, controlled by the coordinated actions of protein tyrosine kinases and phosphatases, is a crucial control mechanism for numerous physiological processes [25, 26]. PEST tyrosine phosphatase is one of the PTPases to regulate such signaling events, although substrate specificities of the PEST tyrosine phosphatase remain to be elucidated [25, 26]. PEST tyrosine phosphatase is known to associate with Shc, and two serine residues at positions 5 and 29 in the amino terminus of Shc are suggested to be the sites regulating binding to the PEST-tyrosine phosphatase

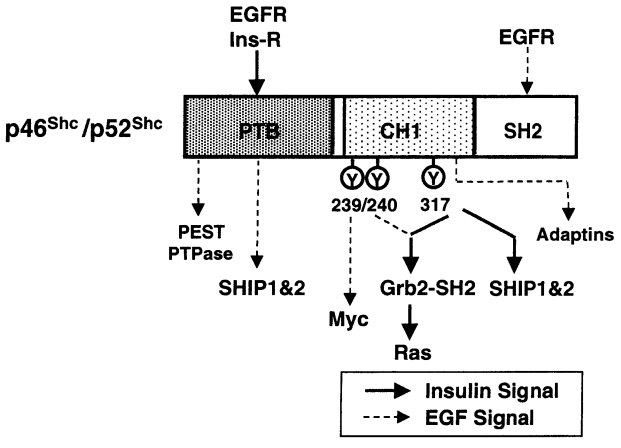

Fig. 4. Representation of Shc interacting molecules. The activated insulin receptor interacts with Shc via Shc PTB domain alone, whereas EGF receptor associates with Shc via both Shc PTB domain and SH2 domain. Upon EGF treatment, the signaling to activate Ras, Myc, PEST protein tyrosine phosphatase, adaptins is mediated via various domains of Shc. On the other hand, insulin mediates Shc·Grb2 pathway via the 317-tyrosine residue of Shc, and competitive interaction of SHIP family for the Shc·Grb2 binding is reported.

[25, 26]. Therefore, binding to Shc may regulate the enzymatic activity of PEST tyrosine phosphatase, although the functional significance of PEST tyrosine phosphatase in insulin signaling is unknown. The adaptins are components of adaptor proteins that anchor the clathrin lattice on the cell surface of coated pits and coated vesicles [27]. Adaptins are assumed to be implicated in receptor endocytosis such as EGF receptors and are shown to constitutively bind to Shc [27]. Amino acids 346–355 in the collagen homology domain of Shc are required for adaptin binding [27]. Dynamin, a 100 kDa protein with GTPase activity, is also thought to play a crucial role in receptor mediated endocytosis [28]. In this regard, dynamin is tyrosine phosphorylated and associates with Shc and insulin receptors upon insulin stimulation, although the precise Shc binding site for dynamin is not characterized [28]. Thus, Shc may be involved in growth factor mediated endocytosis of the insulin receptor and EGF receptor through interaction with dynamin and adaptins, respectively. SH2-containing inositol 5'-phosphatase family composed of SHIP1 and SHIP2 has been shown to associate with Shc [29,30]. Both the SH2 domain and carboxyl-terminal tyrosine residues of SHIP1 and SHIP2 can interact with the Tyr-317 residue and PTB domain of Shc, respectively [29, 30]. In this regard, SHIP2 SH2 domain interaction with Shc may affect insulin-mediated Shc·Grb2 pathway by competing with Shc for binding to Grb2. To clarify the role of the SHIP1 SH2 domain, the WT-SHIP1 and a truncated SHIP1 lacking the SH2 domain (ΔSH2-SHIP1) were expressed into Rat1 fibroblasts overexpressing insulin receptors [29]. As a result, insulin-induced Shc·Grb2 association for MAP kinase activation leading to cell cycle progression was decreased by overexpression of wild-type SHIP1 [29]. In contrast, overexpression of ΔSH2-SHIP1 only minimally affected these events of insulin signaling [29]. SHIP2, which is an isozyme of SHIP1, is abundantly expressed in insulin responsive tissues, and only a small amount of SHIP1 is expressed in these cells [29, 30]. Therefore, the results with SHIP1 overexpression appear to indicate the role of SHIP2. In accordance with this assumption, overexpression of SHIP2 also inhibited insulin-induced Shc·Grb2 association and MAP kinase activation [30]. Therefore, Shc and SHIP2 appear to compete for the limited pool of Grb2 in mediating insulin-

induced mitogenic signaling.

Clinical implication of Shc in diabetes mellitus

Shc has been shown to be a key molecule of growth promotion and gene expression by growth factor receptors including insulin and IGF-1 receptors [2, 23]. Since previous studies have suggested the relationship between intrauterine growth retardation and glucose intolerance or type 2 diabetes occurring later in life, functional abnormalities in Shc protein are considered to be a possible candidate for a certain population of type 2 diabetes [31]. Seventy patients with type 2 diabetes mellitus were screened to examine the genetic abnormality of Shc by single stranded conformation polymorphism-heteroduplex analysis. Subsequent nucleotide sequence analysis demonstrated the existence of Met to Val mutation at codon 300 (numbered according to the 52 kDa Shc; Fig. 1) [31]. This mutation is assumed to exist in all three Shc isoforms [31]. Because this variant is placed in the vicinity of the tyrosine residue at Tyr-317, which is critical for insulin-induced tyrosine phosphorylation of Shc for interaction with Grb2, the impact of the heterozygous mutation of Shc has been considered [31]. However, the allelic frequencies of Met to Val-300 polymorphism in the middle-aged type 2 diabetes and age-matched glucose-tolerant subjects were not significantly different [31]. In addition, the serum insulin and C-peptide responses obtained during the oral glucose tolerance test showed no significant differences between wild-type and heterozygous carriers. Furthermore, insulin sensitivity estimated by Bergman's minimal model and the acute insulin response examined by an intravenous glucose tolerance test in combination with intravenous injection of tolbutamide were not different between heterozygous and wild-type carriers [31]. Although transfection experiments have not been performed to exclude a minor biological impact of this Shc mutation, this mutation of Shc does not appear to be related to the pathogenesis of type 2 diabetes mellitus.

p66 kDa Shc isoform

$p66^{Shc}$ is a splicing isoform of $p52^{Shc}/p46^{Shc}$ and possess a unique amino-terminal CH2 region (Fig. 1) [4]. Like $p52^{Shc}/p46^{Shc}$, $p66^{Shc}$ becomes tyrosine phosphorylated upon activation of EGF receptors, and forms the complex with Grb2 adaptor protein [4]. However, $p66^{Shc}$ does not affect EGF stimulation of MAP kinase activity and inhibits c-fos promoter activation [4]. The role of $p66^{Shc}$ in EGF receptor mediated MAP kinase-fos signaling is in contrast to the effect of $p52^{Shc}/p46^{Shc}$ [4]. In this regard, in addition to tyrosine phosphorylation of the $p66^{Shc}$, $p52^{Shc}$, and $p46^{Shc}$ isoforms, EGF treatment resulted in serine phosphorylation of only the $p66^{Shc}$ isoform [32, 33]. Serine phosphorylation of $p66^{Shc}$ appeared to impair the ability to interact with the EGF receptor and acts in a dominant-interfering fashion by inhibiting EGF receptor downstream signaling pathway [4, 33]. Recently, the role of $p66^{Shc}$ in stress responses has been elucidated [34]. Unlike EGF, $p66^{Shc}$ is not tyrosine phosphorylated by treatment with ultraviolet light (UV) or perhydroxyl oxide (H_2O_2) [34]. However, UV and H_2O_2 as well as EGF provoked phosphorylation of $p66^{Shc}$ on the serine residue [34]. By targeted disruption of $p66^{Shc}$, cells lacking $p66^{Shc}$ were shown to be more viable and protected from apoptosis triggered by treatment with UV or H_2O_2 [34]. As a result, $p66^{Shc}$ null mice are resistant to oxidative stress in vivo, and have increased life span [34]. Although the unique roles of $p66^{Shc}$ have been identified in EGF stimulation and stress responses, insulin treatment does not elicit apparent tyrosine phosphorylation of $p66^{Shc}$ [2, 32]. Based on these results, it is unlikely that $p66^{Shc}$ is directly involved in the regulation of insulin induced MAP kinase activation. However, insulin also stimulates serine phosphorylation of $p66^{Shc}$ but not the $p52^{Shc}$ [35]. Serine phosphorylation of $p66^{Shc}$ appears to be a mechanism dependent on MEK, but not the MAP kinase pathway [35]. Further investigation of the role of $p66^{Shc}$ in insulin signaling and the modulation by serine phosphorylation is needed.

Future prospects of the role Shc in insulin signaling

Recent reports stress the important role of Shc in insulin-induced mitogenic signaling [2, 11]. In contrast, Shc does not appear to be directly involved in metabolic signaling of insulin such as glucose uptake

and glycogen synthesis, while the IRS mediated PI3-kinase pathway plays a key role in such action as insulin's metabolic effects [24]. Although direct involvement of Shc in insulin's metabolic signaling was found to be negligible, Shc may indirectly modify the signaling mediated by the IRS-PI3-kinase pathway. Along this line, a direct interaction between Shc and IRS-1 has been reported in vitro [36]. In addition, Shc-interacted SHIP2 may downregulate PI3-kinase product, because SHIP2 has a 5'-phosphatase activity toward PtdIns(3,4,5)P3 [30]. These possible mechanisms by which Shc regulates IRS-PI3 kinase pathway for metabolic action of insulin remain to be elucidated. Recent studies with knockout mouse have shown interesting results, with the tissue-specific knockout of the insulin receptor in pancreatic β cells leading to a decrease in islet size in the older knockout mice [37]. This finding suggests that insulin signaling leading to islet β-cell growth is related to the amount of insulin for maintenance of glucose homeostasis. In this regard, it is interesting to consider that Shc may be involved in islet β-cell growth. This issue also remains to be elucidated.

Summary

Shc is composed of 46-, 52-, 66-kDa isoforms which arise from alternative splicing of the primary Shc transcript. Upon insulin stimulation, the activated insulin receptor interacts with Shc. The NPXY motif around 960-Tyr residue of the insulin receptor binds to the N-terminal PTB domain of Shc. Subsequently, the 52-kDa, and, to a lesser extent, the 46-kDa Shc isoforms are tyrosine phosphorylated. Although Tyr-239/240 and Tyr-317 residues are the possible candidates of Shc phosphorylation sites, insulin predominantly phosphorylates the Shc Tyr-317 residue. Phosphorylated Shc binds to Grb2 which forms a complex with Sos guanine nucleotide exchange factor for p21ras. Both tyrosine-phosphorylated Shc and IRS can bind to Grb2, but Shc·Grb2·Sos is the predominant coupling pathway from the activated insulin receptor to p21ras. Along this line, microinjection of anti-Shc antibody inhibited insulin-induced mitogenesis, and the guanine nucleotide exchange activity for p21ras is tightly associated with Shc, but not with IRS. On the other hand, insulin only transiently activates p21ras for the strict hormonal regulation. For this regulation, longer time of insulin treatment deactivates p21ras by dissociation of Sos from the Shc·Grb2·Sos complex while Shc is still complexed with Grb2. Thus, Shc plays a critical role in insulin-induced mitogenesis through regulation of p21ras activity. As regards the impact of Shc on the metabolic aspects, Shc is shown to compete with IRS as the substrate of the insulin receptor. Thus, IRS mediated downstream signaling leading to glycogen synthesis was decreased by overexpression of Shc. Taken together, Shc appears to play an important role in insulin induced mitogenesis, whereas Shc may not be required for regulation of the metabolic aspects of insulin.

Acknowledgment

We thank Drs. Hajime Ishihara, Manabu Ishiki, Tsutomu Wada, Hiroyuki Hori, and Shihou Murakami at Toyama Medical & Pharmaceutical University for their assistance in preparing this manuscript.

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