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Insulin Induces Tyrosine Phosphorylation of Shc and Stimulates Shc/GRB2 Association in Insulin-Sensitive Tissues of the Intact Rat

Verónica Páez-Espinosa,[2] Carla R. O. Carvalho,[1] Fernanda Alvarez-Rojas,[2] Luis Janeri,[1] Licio A. Velloso,[1] Antonio C. Boschero,[2] and Mario J. A. Saad[1]

[1]Departamento de Clínica Médica, FCM, UNICAMP, Campinas, SP, Brasil; and [2]Departamento de Fisiologia e Biofísica, Instituto de Biologia, UNICAMP, Campinas, SP, Brasil

Shc is a novel type of tyrosine-phosphorylated protein activated in response to a wide variety of polypeptide ligands. In this study, we used immunoprecipitation and immunoblotting to examine the effect of insulin on Shc tyrosine phosphorylation and Shc/GRB2 association in insulin-sensitive tissues of the intact rat. Following an infusion of insulin, Shc was tyrosine-phosphorylated in the liver, skeletal muscle, and adipose tissue in a time- and dose-dependent fashion, which peaked 5 min after exposure to the hormone and, except in the case of adipose tissue, returned to basal values after 15 min. There was coimmunoprecipitation of Shc and the insulin receptor after stimulation with insulin. Receptor tyrosine kinase activity toward Shc was also observed. Following an infusion of insulin, Shc was found to associate with GRB2. These results demonstrate that after stimulation of rat tissues with insulin, Shc binds to the insulin receptor, is tyrosine-phosphorylated, and subsequently associates with GRB2.

Key Words: Shc; GRB2; tyrosine phosphorylation; tyrosine kinase activity.

Introduction

The insulin receptor (IR) is the principal mediator of insulin action in cellular mitogenic and metabolic processes. The IR β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit *(1–3)*. The discovery of tyrosine kinase activity in the IR suggested that the mechanism of insulin action involves tyrosyl phosphorylation of intracellular substrates *(4,5)*. Using anti-phosphotyrosine antibodies, an insulin-stimulated phosphoprotein called pp185 has been identified in many cells and tissues *(6,7)*. One component of pp185, known as insulin receptor substrate 1 (IRS-1) band, has been purified and cloned from several sources *(7–9)*. Recently, another constituent of the pp185 band termed IRS-2 was also purified, and its cDNA sequence determined *(10)*.

In 1992, Pelicci et al. characterized a cDNA clone that encodes a new protein termed Shc (for *src* homology 2/α-collagen-related). Shc is an ubiquitously expressed cytoplasmic protein, capable of being phosphorylated after stimulation by a wide variety of growth factors and cytokines *(11–14)*. The mammalian *Shc* gene encodes three overlapping proteins of 46, 52, and 66 kDa. In cell cultures, insulin has been shown to induce the phosphorylation of the p46 and p52 isoforms of Shc *(14,15)*. In contrast to IRS-1 and IRS-2, which can associate with a wide variety of downstream effector molecules, the tyrosine phosphorylation of Shc protein leads to a specific association with a small 23-kDa adapter protein, the growth factor receptor-bound protein-2, GRB2 *(16–19)*. GRB2 is constitutively associated with the proline-rich domain of son-of-sevenless (SOS), a guanylnucleotide exchange factor for the p21 GTP-binding protein Ras *(20,21)*.

Once stimulated, the Shc-Grb2/SOS complex induces the activation of a specific mitogenic pathway by removing the mechanism that inhibits initiation of the cell cycle *(13,22,23)*. The latter action represents a crucial step in the transmission of the mitogenic signal within the cell.

Although these findings indicate that Shc has an important role in cellular growth *(24,25)*, there has not been a physiological demonstration of the above pathway of insulin action in animal tissues. For this reason, we have investigated the ability of insulin to stimulate Shc phosphorylation and Shc/GRB2 association in insulin-sensitive tissues of intact rats.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Mario J. A. Saad, Departamento de Clínica Médica, FCM-UNICAMP, Campinas, SP, Brasil, 13081-970.

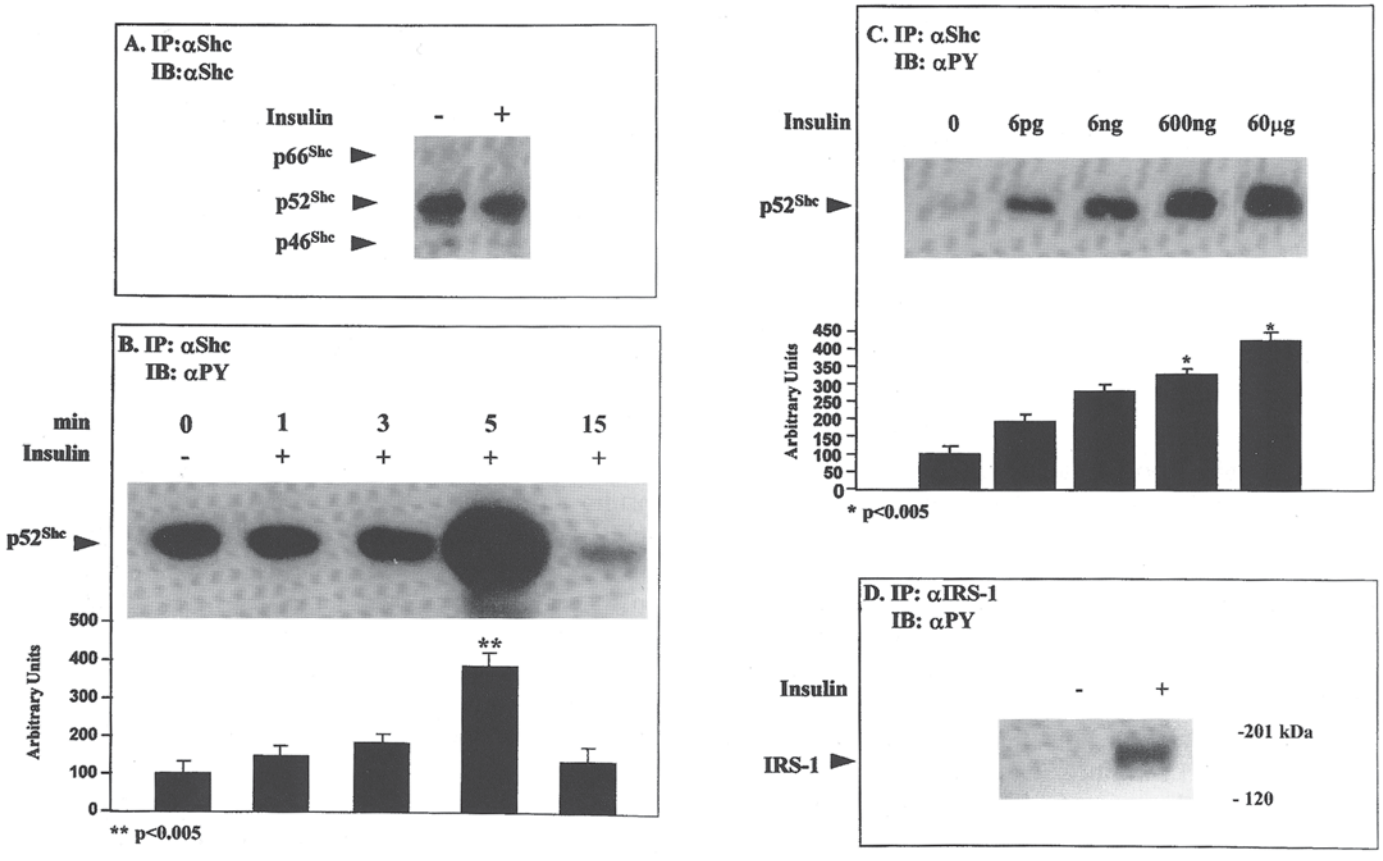

Fig. 1. Shc protein levels **(A)**, time-course **(B)**, and dose–response **(C)** of insulin-stimulated Shc phosphorylation in rat liver. Insulin-stimulated IRS-1 phosphorylation **(D)** in rat liver. Six-week-old male rats were anesthetized and the abdominal wall was incised to expose the viscera. Saline or insulin (at the time and dose indicated) was administered as a bolus injection via the portal vein. In the dose–response experiments, 5 min after insulin infusion, a sample of liver was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. After centrifugation, aliquots from the supernatant were immunoprecipitated with anti-Shc antibody (A, B, and C) or anti-IRS-1 antibody (D) and protein A-Sepharose 6MB, and then resolved on 10% SDS-polyacrylamide gels. The protein bands were subsequently transferred to a nitrocellulose membrane, and detected with anti-Shc antibody (A) or anti-phosphotyrosine antibody (B, C, and D) and ^{125}I-protein A. Scanning densitometry was performed on the autoradiograms of 12 experiments for Shc protein levels, 51 samples from 8 course experiments, and 8 dose–response experiments.

Results

Following stimulation with insulin, Shc was rapidly tyrosine-phosphorylated, regardless of the tissue tested. The predominant isoform observed in liver was p52^{Shc}. In muscle and fat, p52^{Shc} was the only detectable Shc isoform (Figs. 1A, 2A, and 3A, respectively). Using nonimmunized rabbit sera in immunoprecipitation and/or immunoblotting, no bands were detected at 52 kDa.

The effect of insulin on Shc protein levels in liver was examined by immunoprecipitation and immunoblotting techniques, using anti-Shc antibody. As shown in Fig. 1A, there was no change in the level of this protein after acute insulin stimulation.

To estimate the rate of insulin-induced Shc phosphorylation in liver, we performed a time-course experiment after the administration of insulin into the portal vein. Figure 1B shows that there was detectable immunoreactivity in the basal state, indicating that Shc was already tyrosine-phosphorylated before the infusion of insulin in portal vein of fasted rats. Five minutes after the infusion of insulin, there was an increase on Shc tyrosine phosphorylation levels, which declined to basal levels over the following 15 min. An analysis of 51 samples of 8 experiments yielded a mean increase of 380% ($p < 0.005$) in Shc tyrosine phosphorylation 5 min after insulin stimulation.

By immunoblotting the same membranes with anti-Shc antibody, there was no change in the level of Shc protein during the time-course experiments (data not shown).

The insulin-stimulated phosphorylation of Shc in liver was dose-dependent (Fig. 1C). The level of Shc phosphorylation after an infusion of 6 pg of insulin, was nearly half of the maximal stimulation seen with 6 ng of the hormone, and the levels of Shc proteins did not change (data not shown).

We also used the same protocol to perform IRS-1 immunoprecipitation in the rat liver, and compare with Shc phosphorylation levels after insulin infusion. As shown in Fig. 1D, the insulin-induced tyrosine phosphorylation level in liver extracts is higher than the increase in Shc phosphorylation.

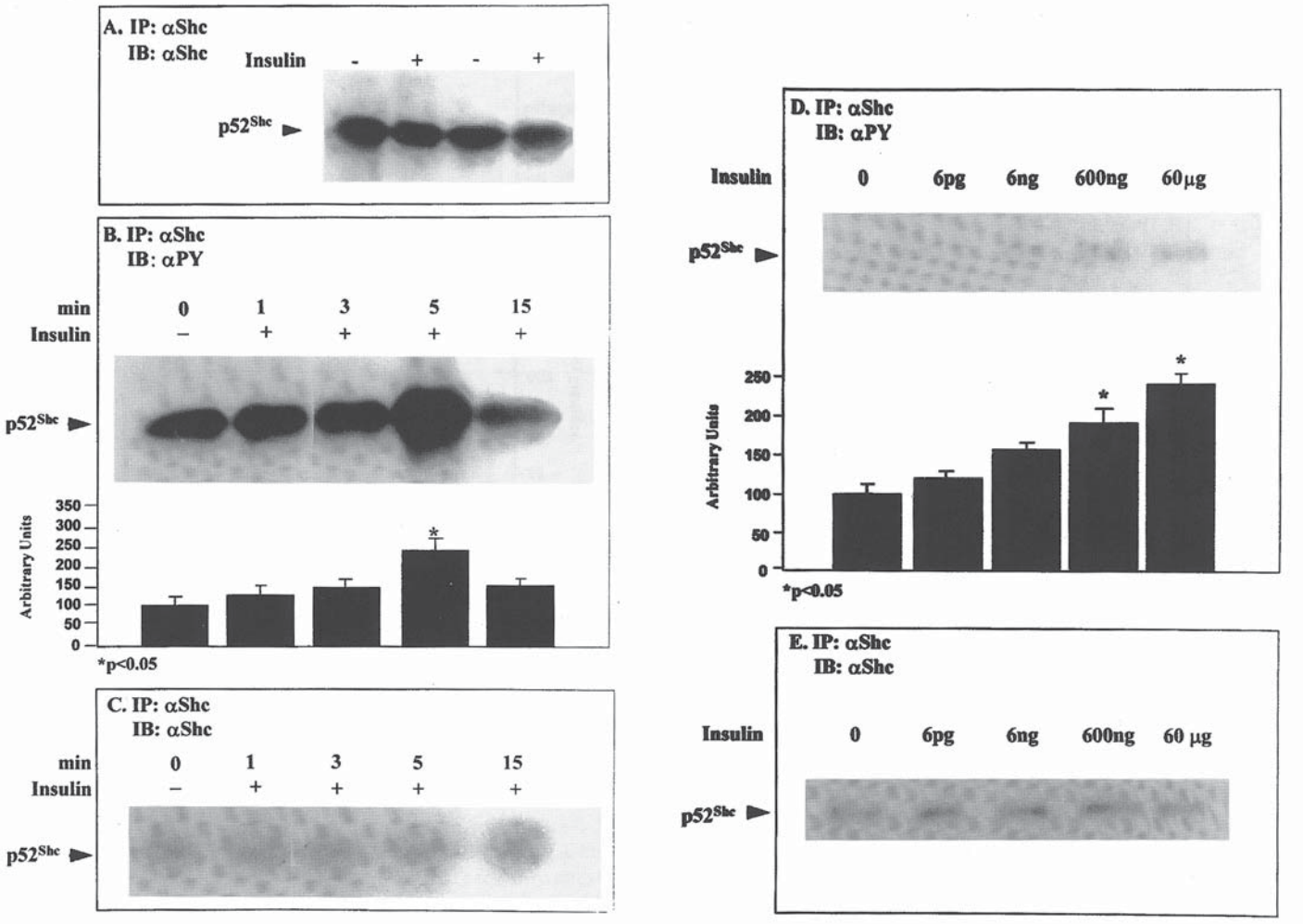

Fig. 2. Shc protein levels **(A)**, time-course **(B** and **C)**, and dose–response **(D** and **E)** of insulin-stimulated Shc phosphorylation in rat muscle. Six-week-old male rats were anesthetized, and the abdominal wall was incised to expose the portal vein. Saline or insulin (at the time and dose indicated) was then administered as a bolus injection. In the dose–response experiments, 5 min after insulin infusion, a sample of a gastrocnemius muscle was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. Muscle samples were immunoprecipitated with anti-Shc antibodies, and immunoblotted with antiphosphotyrosine antibody (B and D) or anti-Shc antibody (A, C, and D). The data are representative of nine experiments for Shc protein levels, 10 time-course experiments, and 7 dose–response experiments.

The time-course of insulin-induced Shc phosphorylation in muscle was similar to that in liver. Some basal immunoreactivity was also detected, indicating that Shc was tyrosine-phosphorylated in rat muscle before insulin stimulation. The level of phosphorylation increased to 232% ($p < 0.05$) at 5 min, returning to basal levels after 15 min (Fig. 2B).

To determine the expression of Shc in muscle during the time-course, we studied the Shc protein levels before and after insulin injection. Figure 2C shows that there was no change in protein levels following the administration of the hormone.

The insulin-induced phosphorylation of Shc in muscle was dose-dependent (Fig. 2D). The presence of phosphorylated Shc was detectable after the injection of 6 pg of insulin. Half-maximal stimulation of this phosphorylation was obtained with an insulin dose between 6–600 ng. Shc protein levels in dose–response experiments remained unchanged (Fig. 2E).

The time-course of insulin-induced Shc tyrosil phosphorylation in adipose tissue was slightly different from that of liver and muscle. In adipose tissue, Shc phosphorylation increased to 204% 1 min after insulin infusion, and reached a maximum at 280% ($p < 0.05$) 5 min later. However, the level of phosphorylation was sustained for at least 15 min (Fig. 3B).

As with liver and muscle, there was no change in the levels of Shc protein in adipose tissue after insulin stimulation during the time-course experiments (Fig. 3C).

The insulin-stimulated phosphorylation of Shc in adipocyte extracts was dose-dependent. The levels of phosphorylated Shc reached a half-maximum value with 6 pg of insulin (Fig. 3D). When these membranes were blotted with anti-

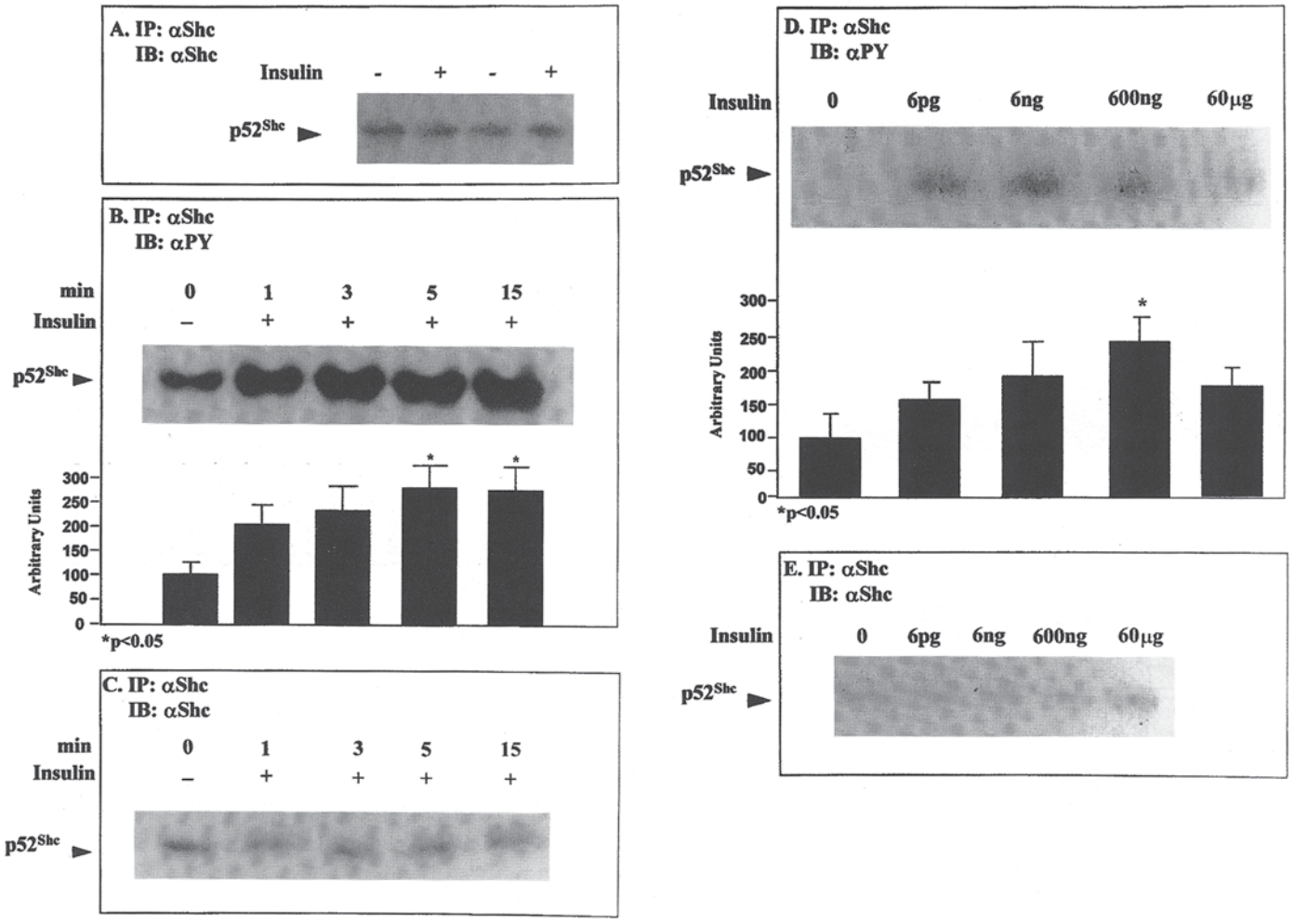

Fig. 3. Shc protein levels (**A**), time-course (**B** and **C**), and dose–response (**D** and **E**) of insulin-stimulated Shc phosphorylation in rat adipose tissue. Six-week-old male rats were anesthetized, and the abdominal wall was incised to expose the portal vein. Saline or insulin (at the time and dose indicated) was administered as a bolus injection via the portal vein. In the dose–response experiments, 5 min after insulin infusion, a sample of adipose tissue was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. Adipose tissue samples were immunoprecipitated with anti-Shc antibodies, and immunoblotted as described in legend for Fig. 2. The data are representative of 6 experiments for Shc protein levels, 9 time-course experiments, and 8 dose–response experiments.

Shc antibody, the levels of Shc protein remained unchanged (Fig. 3E).

The rapid induction of Shc tyrosine phosphorylation by insulin suggested that Shc may associate with the insulin receptor. To test this possibility, liver extracts were immunoprecipitated with anti-Shc antibody before and after insulin stimulation, and the precipitated proteins then probed with antireceptor antibody. Shc was found to coprecipitate with the insulin receptor after stimulation with the hormone (Fig. 4A). Anti-insulin receptor coimmunoprecipitation was also performed with the same tissue extracts. As shown in Fig. 4B, Shc was coimmunoprecipitated by anti-insulin receptor antibody following insulin stimulation.

To determine whether insulin-induced insulin receptor kinase activity was able to phosphorylate Shc, we measured enzyme autophosphorylation and kinase activity in vitro. Following the infusion of saline or a low dose of insulin (6 pg) into the portal vein to obtain limited tyrosine phosphorylation of the insulin receptor, liver extracts were immunoprecipitated with antibody to the insulin receptor. The pellet was incubated at room temperature with ATP to permit IR autophosphorylation. Figure 4C demonstrates that after infusion of a low dose of insulin, there is a limited autophosphorylation of insulin receptor and also a discrete increase in tyrosine phosphorylation of a lower band (~55 kDa). After infusion of a low dose of insulin in vivo and the addition of ATP in vitro, there is a clear increase in insulin receptor autophosphorylation and in tyrosine phosphorylation of at least four bands (the same lower-molecular-mass band and three bands higher than insulin receptor). By stripping and immunoblotting the same membrane with specific antibodies, we demonstrate that the two higher bands are IRS-2 and IRS-1, and that the lowest band of ~55 kDa is Shc. We have not identified the band appearing between IRS-1 and insulin receptor band.

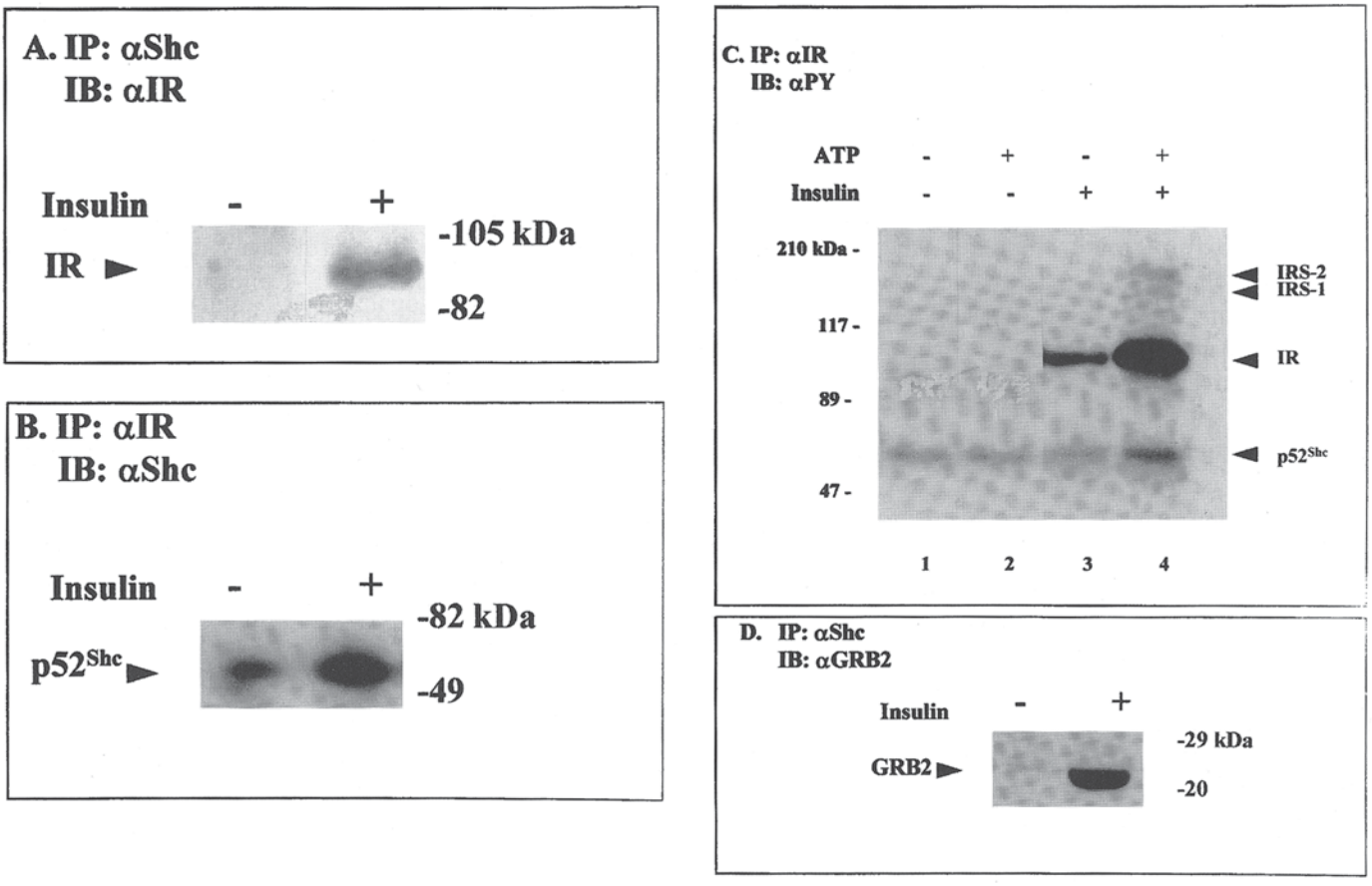

Fig. 4. The association of Shc with the insulin receptor (**A** and **B**), IR tyrosine kinase activity measured by autophosphorylation **(C)**, and Shc-GRB2 association in rat liver **(D)**. The rats were anesthetized and the abdominal wall was incised to expose the viscera. Saline or 60 μg of insulin (as indicated in the figure) were administered as a bolus injection into the portal vein. Five minutes later, a sample of the tissue were excised and homogenized in extraction buffer at 4°C as described in Materials and Methods. After centrifugation, aliquots containing equal amounts of protein were immunoprecipitated (IP) with anti-Shc or anti-insulin receptor (αIR) antibodies as indicated in the figure. The immunoprecipitates were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then immunoblotted (Blot) sequentially with anti-insulin receptor (A), anti-Shc (B), or anti-GRB2 (D) antibodies as indicated in the figure. The data are representative of five experiments for each antibody. To measure the tyrosine kinase activity of IR (C), 6 pg of insulin were injected into the portal vein of the rat, and the liver was excised 30 s (the maximal time of IR tyrosine phosphorylation in liver) after the insulin injection. To stimulate partial IR autophosphorylation, IR was then immunoprecipitated and allowed to autophosphorylate in vitro in the presence of exogenous ATP (lane 4). Tyrosine phosphorylation was measured by immunoblotting the ATP stimulated pellet with an antiphosphotyrosine antibody. Control conditions are shown in lanes 1 and 2. In lane 1, the liver extract was not exposed to insulin or ATP. In lane 2, the liver extract was not exposed to insulin, but ATP was added to the in vitro autophosphorylation reaction. In lane 3, insulin (6 pg) was infused into the portal vein and the liver extracted after 30 s, but no exogenous ATP was added during the in vitro phosphorylation step.

To determine the presence of Shc/GRB2 association in liver extracts, samples of this tissue were immunoprecipitated with anti-Shc antibodies before and 5 min after insulin stimulation, and blotted with anti-GRB2 antibodies. Shc/GRB2 association was observed only when the Shc protein was phosphorylated (Fig. 4D).

Discussion

It is well established that the hormone-stimulated tyrosine kinase activity of growth factor receptors is a necessary event in the propagation of growth factor-dependent downstream signaling *(26–30)*. Although several combinations of proximal effectors have been identified for numerous growth factor tyrosine kinase receptors, they all appear to have in common tyrosine phosphorylation and/or association with Shc proteins *(11,15,18)*. Insulin has also been shown to phosphorylate this protein in cultured cells *(11,14,15)*, although the physiological significance of this process has not yet been elucidated. We have demonstrated here that in liver, skeletal muscle, and, to a lesser extent in adipose tissue, Shc undergoes tyrosine phosphorylation after insulin administration in intact rats.

Di Guglielmo et al. *(31)* demonstrated in rat liver that epidermal growth factor (EGF) administration clearly induced Shc tyrosine phosphorylation, whereas insulin infusion led to insulin receptor tyrosine phosphorylation,

but little detectable tyrosine phosphorylation of Shc and no recruitment of GRB2. In the study of Di Guglielmo et al., preparative subcellular fractionation was used to address signal transduction in vivo, whereas our work experiments were conducted in whole-tissue extracts. In addition, the composition of homogenization buffers we used was different. Thus, in our studies, the concentration of vanadate was 100-fold greater than that used in the studies of Di Guglielmo et al. These methodological differences might explain why Di Guglielmo et al. found little detectable Shc tyrosine phosphorylation, whereas we observed a moderate increase in Shc tyrosine phosphorylation in liver after insulin stimulation.

In vitro binding experiments have shown that the 52-kDa Shc isoform associates more efficiently with the insulin receptor than the 46-kDa Shc isoform *(14,15)*. In previous studies using cultured cells, the 52-kDa Shc isoform was also shown to have a higher level of tyrosine phosphorylation than the 46-kDa species when stimulated with insulin *(14,15,23)*. Our results for rat muscle and adipose tissue demonstrate that after treatment with insulin, only the 52-kDa Shc isoform was tyrosine-phosphorylated. In liver, this isoform of the protein presents high levels of phosphorylation with little phosphorylation of the 66- and 46-kDa isoforms. This predominance may be a consequence of the high levels of the 52-kDa Shc isoform in rat tissues compared with other isoforms, although interactions with the insulin receptor may also play a role in this pattern of phosphorylation.

To our knowledge, this is the first demonstration of insulin-induced Shc tyrosine phosphorylation in intact animals. Acute, half-maximal stimulation of Shc phosphorylation in liver occurred after the infusion of 6 ng of insulin, which corresponds to plasma insulin levels of 40 μU/mL, 1 min later (data not shown). This concentration is similar to that which is achieved in rats during an oral glucose or meal tolerance test. Shc tyrosine phosphorylation in muscle and adipocytes following the intraportal injection of insulin was less sensitive than in liver, probably because of the lower peripheral insulin concentrations.

The insulin-induced Shc phosphorylation was similar to that reported for EGF and platelet-derived growth factor (PDGF) signaling *(14,15,32)*. Shc proteins have also been shown to associate with the activated EGF receptor *(11,33–35)*. Although previous studies in cell cultures found no association between Shc proteins and the activated insulin receptor, we have shown that these two proteins coimmunoprecipitate after an infusion of insulin. Recent studies have demonstrated that Shc must bind directly to the activated insulin receptor via its PTB/PI domain in order to be tyrosine-phosphorylated in vivo *(10,36–38)*. Studies using cells expressing insulin receptor mutants support a model in which both Shc and IRS-1 recognize a core NPX-Tyr(P) motif on the insulin receptor, with the amino acids surrounding this motif being critical for binding either IRS-1 or Shc *(39)*. Our results also showed that insulin receptor has tyrosine kinase activity toward Shc after insulin stimulation.

Both Shc and IRS-1 are phosphorylated at similar insulin concentrations, but maximal Shc phosphorylation occurs later than IRS-1 maximal phosphorylation in rat tissues *(40,41)*. We have previously demonstrated that maximal insulin-induced IRS-1 phosphorylation occurs at 30 and 90 s in liver and muscle, respectively *(40,41)*. The present study is showing that in both tissues, maximal insulin-induced Shc tyrosine phosphorylation occurs at 5 min postinjection of insulin. The physiological consequence of this differential time-course is not known, but since Shc and IRS-1 compete for the same binding site at the insulin receptor, it is tempting to speculate that the receptor uses different substrates at different times, in order to serve as a more effective tyrosine kinase toward each substrate. It is also interesting that IRS-1, which is phosphorylated first, mediates the metabolic and growth-promoting effects of insulin, whereas Shc, which is phosphorylated later, is mainly a component of growth-signaling pathways.

In cultured cells, insulin-induced tyrosine phosphorylation of Shc is followed by the association of Shc with a small 23-kDa adapter protein known as GRB2 *(16–19)*. Our demonstration that Shc associates with GRB2 in rat tissues after insulin stimulation supports the idea that tyrosine phosphorylation of Shc, and the subsequent interaction of this protein with GRB2, may be an essential component of a common pathway used by many tyrosine kinases. The Shc/GRB2 interaction may play a crucial role in the activation of $p21^{ras}$ and in the control of downstream effector molecules *(22)*.

It is interesting that in Chinese hamster ovary cell lines with mutations at two tyrosine phosphorylation sites on the insulin receptor there is a dissociation between insulin-induced IRS-1 and Shc tyrosine phosphorylation *(42,43)*. These cell lines show normal insulin-induced tyrosine phosphorylation of Shc, Shc–GRB2 complex formation, and $p21^{ras}$ activation, but have reduced tyrosine phosphorylation of IRS-1, as well as reduced IRS-1 association with GRB2 and PI3-kinase *(42)*. A dissociation between IRS-1 and Shc tyrosine phosphorylation has also been observed in other cell lines, without any change in the insulin receptor phosphorylation level, following treatment with dexamethasone *(44)* or wortmannin *(45)*. In this regard, it would be of interest to investigate, in parallel insulin-induced insulin receptor, IRS-1, and Shc tyrosine phosphorylation in the tissues of animal models of insulin resistance.

In summary, we have shown that following the stimulation of rat tissues with insulin, Shc binds to the insulin receptor, is tyrosine-phosphorylated, and subsequently associates with GRB2.

Materials and Methods

Materials

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PSMF), aprotinin, silicone, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly Co. (Indianapolis, IN). Adenosine 5'-triphosphate (ATP) from equine muscle was from Sigma Chemical Co. Protein A-Sepharose 6 MB was purchased from Pharmacia (Uppsala, Sweden). [^{125}I] protein A was obtained from Amersham (Aylesbury, UK), and nitrocellulose (BA85; 0.2 μm) was obtained from Schleicher and Schuell (Keene, NH). Male Wistar rats were from the UNICAMP Central Animal Breeding Center. Monoclonal antiphosphotyrosine antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Shc, anti-GRB2, anti-IRS-1, and anti-insulin receptor antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Six-week-old male Wistar rats (mean body wt of 131 g ± 6 g) were used. The animals were fed standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments (except for animals used to determine IR tyrosine kinase activity, which were fasted for only 4 h). All animal experiments were approved by the Ethical Committee of the State University of Campinas (UNICAMP).

Methods

The rats were anesthetized with sodium amobarbital (15 mg/kg body wt, ip) and used 10–15 min later, as soon as anesthesia was assured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 mL of saline (0.9% NaCl) with or without 6 μg of insulin (or the concentration described for the dose–response experiments) was injected. Samples of liver, skeletal muscle, and adipose tissue were collected at 0, 1, 3, 5, and 15 min after insulin infusion, minced coarsely, and homogenized immediately in approx 10 vol of solubilization buffer A at 4°C, using a Polytron PTA 20Ss homogenizer (Brinkmann Instruments, model PT 10/35), operated at maximum speed for 30 s. Buffer A (for solubilization) consisted of 1% Triton X-100, 50 m*M* HEPES (pH 7.4), 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* EDTA, 10 m*M* sodium vanadate, 2 m*M* PSMF, and 0.1 mg of aprotinin/mL. The extracts were centrifuged at 30,000*g* in a Beckman 70.1 Ti rotor at 4°C for 20 min in order to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with 15 μL of polyclonal anti-Shc. The immune complexes were precipitated with protein A-Sepharose 6MB and were washed three times with 50 m*M* Tris, pH 7.4, 2 m*M* sodium vanadate, and 0.1% Triton X-100.

Protein Analysis by Immunoblotting

After washing, the pellet was suspended in Laemmli sample buffer with 100 m*M* dithiothreitol and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (10% Tris-acrylamide) in a Bio-Rad miniature slab gel apparatus.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. *(46)*. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (3% BSA, 10 m*M* Tris, 150 m*M* NaCl, and 0.02% Tween-20). The prestained molecular-mass standards used were phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.6 kDa), and lysozyme (19.4 kDa). The nitrocellulose filter thus treated was then incubated for 4 h at 22°C with antiphosphotyrosine antibody, anti-insulin receptor antibody, or GRB2 antibody (0.5 μg/mL each, diluted in blocking buffer), and then washed for 30 min in blocking buffer without BSA. The blots were then incubated with ^{125}I-protein A (30 μCi/μg) in 10 mL of blocking buffer for 1 h at 22°C and washed again. ^{125}I-protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at –70°C for 12–48 h. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

IR Tyrosine Kinase Activity Toward Shc

Following the infusion of a very low dose of insulin (6 pg) or saline into the portal vein of 4-h fasted rats, liver extracts were immunoprecipitated as described above, using anti-insulin receptor (β-subunit) antibody. The immune complexes were collected on protein A/G plus.

The protein kinase activity of the immunoprecipitates was measured by incubating the immune complexes (anti-insulin receptor) in 100 μL of buffer containing 50 m*M* Tris, pH 7.5, 0.2 m*M* sodium vanadate, 0.1% Triton X-100, 3 m*M* $MnCl_2$, and 15 μ*M* ATP for 30 min at room temperature. The complexes were washed twice with cold buffer, and then suspended in Laemmli sample buffer and analyzed by SDS-PAGE according to the method of Laemmili *(47)*. The separated proteins were transferred to nitrocellulose membranes. The incorporation of phosphate into insulin receptor and other proteins that might be substrates bound to IR was visualized by autoradiography of antiphosphotyrosine immunoblots *(48)*.

Statistical Analysis

Time-course and dose–response data are presented as the mean ± SE of *n* experiments for liver, muscle, and adipose tissue. To check the fit of data for the assumption of parametric analysis of variance, Bartlett's test for the

homogeneity of variances was used. When necessary, the data were log-transformed to correct for variance heterogeneity or nonnormality *(49)*. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey (equal *n*) or Tukey-Kramer (unequal *n*) test for individual differences between times or insulin concentrations. Protein Shc levels were analyzed by Student's *t*-test. The level of significance employed was $p < 0.05$.

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