Effect of nutritional state on the formation of a complex involving insulin receptor IRS-1, the 52 kDa Src homology/collagen protein (Shc) isoform and phosphatidylinositol 3'-kinase activity

Joëlle DUPONT, Michel DEROUET, Jean SIMON and Mohammed TAOUIS¹

Institut National de la Recherche Agronomique, Station de Recherches Avicoles, Endocrinologie Moléculaire et cellulaire du Métabolisme, Centre de Tours, Nouzilly 37380, France

The Src homology and collagen protein (Shc) is tyrosine phosphorylated in response to insulin; however, evidence for its interaction with insulin receptor (IR) in normal tissues is missing. Interactions between IR, Shc and regulatory subunits of the phosphatidylinositol 3'-kinase (PI 3'-kinase) were characterized in the present study in liver and muscles of chickens submitted to various nutritional states. A chicken liver Shc cDNA fragment encoding a 198 amino acid long fragment, including the phosphotyrosine binding domain was sequenced. It shows 89% homology with the corresponding human homologue. The amounts of the three Shc isoforms (66, 52 and 46 kDa) and Shc messenger were not altered by the nutritional state. Shc tyrosine phosphorylation was decreased by fasting in both liver and muscle. Importantly, Shc was immunoprecipitated by IR antibody (mostly the 52 kDa isoform) or by α IRS-1 (mostly the

46 kDa isoform). IR–Shc association was decreased by fasting and restored by refeeding. In liver, α Shc immunoprecipitated the three forms of regulatory subunits of PI 3'-kinase and a PI 3'-kinase activity which was decreased by fasting. In muscle, α Shc immunoprecipitated only the p85 isoform; the associated PI 3'-kinase activity was not altered by the nutritional state. Conversely, in both tissues anti-p85 antibody precipitated only the 52 kDa Shc isoform. In liver, antibodies to insulin receptor substrate-1 (α IRS-1), Shc or IR immunoprecipitated the three regulatory subunits of PI 3'-kinase and an equal PI 3'-kinase activity, without any residual activity left in the supernatants, suggesting the presence of a large complex involving IR, IRS-1, Shc (mainly the 52 kDa isoform) and PI 3'-kinase activity. The presence of another complex containing IRS-1 and the 46 kDa Shc isoform, but no PI 3'-kinase activity, is suggested.

INTRODUCTION

Upon insulin binding, the insulin receptor (IR) autophosphorylates and phosphorylates various cellular substrates on tyrosine residues. At the moment, five of these substrates have been identified. IRS-1 (where IRS is IR substrate) is ubiquitous and is considered to be the major IR substrate in mammalian cells [1]. IRS-2, identified after IRS-1 invalidation in mice, corresponds to 4PS, the substrate for interleukin-4 [2,3]. IRS-3 and IRS-4 have been identified recently in rat adipocytes and human kidney respectively [4,5]. The Src homology and collagen protein (Shc), which is involved in the mitogenic signalling of a variety of tyrosine kinase growth-promoting receptors, such as epidermal growth factor-R (EGF-R), platelet-derived growth factor-R and nerve growth factor-R, through the *ras/raf* cascade [6–10], is also phosphorylated on tyrosine residues in response to insulin [11]. IRS-1–4 proteins contain several tyrosine residues located in specific motifs (YXXM, YMXM). Phosphorylation of these tyrosine residues permits their interaction with the Src homology domain 2 (SH2) of various signal-transducing molecules, such as the regulatory subunit p85 of the phosphatidylinositol 3'-kinase (PI 3'-kinase), Grb2, the phosphatase SH-PTP2 (Syp), Nck and Fyn [12,13]. The Shc proteins include three isoforms that are encoded by the same gene: 66, 52 and 46 kDa isoforms [10]. Shc isoforms and IRS-1 have in common phosphotyrosine binding (PTB) and plekstrin domains which are crucial for the interaction with phosphorylated IR. Thus, the molecular

organization of Shc and IRS-1 appears to be similar. Shc also possesses a glycine–proline-rich region [collagen homology 1 (CH1)], which interacts with proteins containing SH3 domain [10,14,15]. Another CH domain called CH2 has been described in the N-terminal end of a 66 kDa isoform [16]. The phosphorylation of Shc by the IR has been reported in different transformed-cell models. IR appears to phosphorylate the 52 kDa isoform preferentially, whereas EGF-R phosphorylates the three Shc isoforms [17,18]. However, to our knowledge, the interaction between Shc and IR has been demonstrated only *in vitro* [19–21] and its existence in normal cells and in tissues is still a matter of debate [11,22,23].

We have recently cloned and sequenced the chicken IRS-1 gene [24]. Chicken IRS-1 structure and deduced amino acid sequence are very conserved. During studies aimed at characterizing IRS-1 phosphorylation in a chicken cell line [25] and *in vivo* [26], another protein potentially related to Shc was also revealed to be phosphorylated on tyrosine residues. Therefore, this animal species is potentially a useful tool in the study of Shc–IR interactions in normal tissues. In the present studies, it is shown that: (1) the three Shc isoforms are present in chicken liver and muscles; (2) Shc, mainly the 52 kDa isoform, associates with IR; (3) in both tissues, the tyrosine phosphorylation state of Shc is dependent upon the nutritional state (and therefore most likely plasma insulin levels); (4) the presence of a large complex involving IR, IRS-1, Shc isoforms, the regulatory subunits of PI 3'-kinase, as well as a PI 3'-kinase activity, is demonstrated; and

Abbreviations used: ECL, enhanced chemiluminescence; PI 3'-kinase, phosphatidylinositol 3'-kinase; IR, insulin receptor; IRS, insulin receptor substrate; Shc, Src homology and collagen protein; PTB, phosphotyrosine binding; CH, collagen homology; SH2, Src homology 2; SH3, Src homology 3; EGF, epidermal growth factor; RT-PCR, reverse transcription PCR.

¹ To whom correspondence should be addressed (e-mail taouis@tours.inra.fr).

(5) a smaller complex including Shc (mainly the 46 kDa isoform) and IRS-1, but no PI 3'-kinase activity, is also present.

EXPERIMENTAL

Chemicals

[¹²⁵I]Iodine was purchased from Amersham France (Les Ulis, France) and [γ -³²P]ATP was obtained from Isotopchim (Peyruis, France). BSA (fraction V, radioimmunoassay grade), PMSF, leupeptin, aprotinin and Protein A-agarose and phosphatidylinositol were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Triton X-100, SDS and nitrocellulose membrane were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and pre-made polyacrylamide solution protogel was from Prolabo (National Diagnostics, France). Silica TLC plates were obtained from Carlo Erba (France). Nylon membranes (hybond N+) were from Amersham (Les Ulis, France). The Quick Prep total RNA extraction kit, dNTP and Taq DNA polymerase were purchased from Pharmacia (France). Avian myeloblastosis virus reverse transcriptase (AMV) and RNase ribonuclease inhibitor (rRNasin) were purchased from Appligen-Oncor (France). Random Hexamer primers were purchased from Promega (France). Primers used for reverse transcription PCR (RT-PCR) were provided by Gibco-BRL, Life Technologies (Cergy-Pontoise, France) and by Genosys (UK).

Antibodies

Antibodies to mouse Shc (α Shc) and antiphosphotyrosines (α PY20) were from Transduction Laboratories (Ozyme, France). Antibodies to mouse IRS-1 were from UBI (Lake Placid, NY, U.S.A.) and Transduction Laboratories (Lexington, KY, U.S.A.). Antibodies to mouse p85 regulatory subunit of PI 3'-kinase (α p85) were purchased from UBI (Lake Placid). Antibodies to insulin receptor (B10), a serum from an autoimmune patient, was a generous gift from Dr. Ph. Gorden (NIDDK, NIH, Bethesda, MD, U.S.A.) and antibodies to mouse EGF receptor from Oncogene Science were a gift from Dr. H. Khatir (INRA, Nouzilly, France).

Animals

Male chickens (broiler-type, $n = 18$) were bred in a conventional floor pen, fed *ad libitum* with a balanced diet [3100 kcal/kg metabolizable energy and 22% protein (1 cal = 4.184 J)] and exposed to a daily 14 h light period. At 9 weeks of age, animals were separated into three groups and submitted to three different nutritional states: (1) fasted for 48 h; (2) refed for 30 min after 48 h fast; and (3) fed *ad libitum*. After blood sampling and cervical dislocation, tissues (liver and leg muscles) were quickly removed, frozen in liquid nitrogen, ground in powder and stored at -80°C until used.

Determination of plasma glucose and insulin levels

Plasma glucose levels were measured by the glucose oxidase method (Glucose Beckman Analyser 2, Palo Alto, CA, U.S.A.) and plasma insulin levels by RIA with a guinea pig anti-porcine insulin antibody (Ab 27-6; generously provided by Dr. G. Rosselin, Hôpital Saint-Antoine, Paris, France) using chicken insulin as the standard.

Immunoprecipitations and Western-blotting procedures

Powdered tissues (1 g) were homogenized, solubilized and ultracentrifuged as previously described [26]. The supernatants were

immunoprecipitated with different antibodies: α Shc (1/200), α PY20 (1/200), B10 (1/100), α EGF receptor (1/100) or α IRS-1 (1/100) at 4°C for a period of 16 h. The immune complexes were precipitated with Protein A-agarose for 1 h at 4°C as previously described [27]. Immunoprecipitates were resolved by SDS/PAGE and transferred to nitrocellulose membranes. Blots were blocked and probed with α PY20, α p85 or α Shc at a dilution of 1/500. Immune complexes were detected with horseradish peroxidase conjugated with specific secondary antiserum (Pierce), followed by an enhanced chemiluminescence (ECL) reaction. Band densities were estimated by using the NIH Image Software. When Western blotting was performed without immunoprecipitation, liver and muscle extracts (125 μg of protein) were directly subjected to SDS/PAGE.

PI 3'-kinase assay

PI 3'-kinase was determined as previously described [28,29]. Briefly, the liver and muscles were homogenized, solubilized and ultracentrifuged. The supernatants were immunoprecipitated overnight at 4°C with different antibodies: α Shc (1/200), α PY20 (1/200), α IRS-1 (1/100) or B10 (1/100). PI 3'-kinase was measured in different immunoprecipitates in the presence of phosphatidylinositol and labelled ATP. The radioactivity incorporated into phosphatidylinositol was quantified with a STORM apparatus (Molecular Dynamics).

Determination of chicken Shc expression using RT-PCR; cloning and sequencing of the chicken cDNA coding for the Shc PTB domain

Total RNAs from liver and muscles were extracted using Quick Prep Total RNA Extraction kit according to manufacturer's recommendations (Pharmacia). Total RNAs were subjected to RT-PCR as previously described [24]. RT reactions were primed with random hexamer primers and PCR was carried out in the presence of two pairs of primers. The first pair was chosen from the mouse Shc 52 gene published sequence [7]: sense 5'-CGGG-AGCTTTGTCAATAAGCC3' and antisense: 5'-GAGCCATC-AAAGCCAGCCATC3', flanking a 594 bp region including the PTB domain. The second pair of primers was specific to the cDNA from mouse 18 S rRNA cDNA, sense: 5'-CTGCCCTA-TCAACTTTTCG3' and antisense: 5'-CATTATTCTAGCTG-CGG3', flanking a 515 bp region.

The 594 bp amplified chicken cDNA fragments were cloned using the pCR Script Amp SK(+) cloning kit (Stratagene, La Jolla, CA, U.S.A.) and automatically sequenced using an ABI automated Sequencer (Perkin-Elmer, France).

Southern blot

After RT-PCR and electrophoresis, amplified products were transferred onto Nylon membranes. The blots were hybridized using 6×10^6 c.p.m. of γ -³²P-labelled primer using T4 polynucleotide kinase. Prehybridization was carried out in a buffer containing 50% (v/v) formamide, $5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M sodium citrate), 50% (v/v) 10 $\times$ Denhardt's solution (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA), 1% (w/v) SDS and 50 mM Tris/HCl, pH 7.5, for 4 h at 55°C . Hybridization was assessed overnight at 55°C . Autoradiography was carried at -70°C for 16 h using Kodak X-OMAT film with intensifying screens. Membrane-incorporated radioactivity was also quantified using a STORM apparatus (Molecular Dynamics).

Statistical analysis

Statistical analyses were performed using analysis of variance to detect significant intergroup differences (fed *ad libitum*, fasted for 48 h and refed for 30 min after the fasting period). When a significant value of *F* was found after analysis of variance, the individual treatments were compared by Newman–Keuls test. PI 3'-kinase activities from multiple immunoblots were expressed in arbitrary densitometry units after normalization, using the mean value of samples from chickens fed *ad libitum* as a control within each experiment. Comparisons between fasted (48 h) and refed (30 min after 48 h of fasting) groups were made using Student's *t*-test. All results are expressed as the mean $\pm$ S.E.M. and *P* < 0.05 was considered statistically significant.

RESULTS

Effect of nutritional state on plasma glucose and insulin levels and on liver IR number

In order to modify plasma insulin levels, animals were submitted to three different nutritional states: fed *ad libitum*, fasted for 48 h and refed for 30 min after fasting for 48 h. Fasting induced a significant decrease in plasma insulin level by at least 2-fold when compared with the fed state (Table 1), whereas refeeding increased it by about 5-fold when compared with the fasted state and by 3-fold when compared with the fed state (Table 1). As expected, the variation in plasma insulin levels induced changes in specific insulin binding in liver membranes. Fasting induced a significant up-regulation of IR number *in vivo* (*P* < 0.05; Table 1). Refeeding for 30 min initiated the reversal of this effect, although with heterogeneity among animals.

Characterization of Shc in chicken liver and muscle *in vivo*

To determine whether the three Shc isoforms described in mammals are present in chickens, liver and muscle homogenates from chickens subjected to the three nutritional states were immunoprecipitated with α Shc and immunoblotted with the same antibody after SDS/PAGE and electrotransfer. Three bands were revealed in both muscle and liver (Figure 1, upper panel); they exhibited apparent molecular masses of 66, 52 and 46 kDa, which correspond to those of mammalian Shc isoforms. The 52 and 46 kDa isoforms are predominant in both tissues. Furthermore, the level of the three isoforms, expressed as a percentage of the level observed with the fed state, was not altered by the nutritional state in liver (refed = 106 ± 4.3 and fasted = 106.7 ± 2.2 ; *n* = 3 per nutritional state) and in muscle (refed = 96 ± 4.5 and fasted = 101.7 ± 3.9 ; *n* = 3). The level of Shc tyrosine phosphorylation was assessed in liver and muscle

Table 1 Effect of various nutritional states on plasma glucose and insulin levels and liver membrane insulin binding

Results are given as means $\pm$ S.E.M.; *n* = 6 for plasma glucose and insulin levels and *n* = 4 for liver membrane insulin binding. Blood samples were taken from 9-week-old male chickens which had been fasting for 48 h, fed *ad libitum* or refed for 30 min after a 48 h fasting period. Insulin binding was measured on liver membranes. Results are expressed as specific tracer binding, B_0/T (%). The same letter indicates no significant differences between the various nutritional states (Newman–Keuls test).

Nutritional state	Fasted	Fed <i>ad libitum</i>	Refed
Glucose (g/l)	2.62 $\pm$ 0.03 a	2.68 $\pm$ 0.06 a	3.03 $\pm$ 0.06 b
Insulin (ng/ml)	0.64 $\pm$ 0.15 b	1.19 $\pm$ 0.13 a	3.10 $\pm$ 0.21 c
Insulin binding (B_0/T , %)	15.20 $\pm$ 0.70 b	10.30 $\pm$ 0.70 a	12.95 $\pm$ 1.50 b

Figure 1 Shc expression and tyrosine phosphorylation in chicken liver and muscle

Liver (lanes 1, 2, 3) and muscle (lanes 4, 5, 6) samples from chickens fed *ad libitum* (lanes 1, 4), refed (lanes 2, 5) or fasted (lanes 3, 6) were isolated and solubilized as described in the Experimental section. Samples were immunoprecipitated with either α Shc (upper panel) or α PY20 (lower panel). After SDS/PAGE and electrotransfer, membranes were blotted with α Shc and bands were revealed by ECL. Apparent molecular masses (in kDa) are indicated on the left side of the Figure. Similar experiments were repeated with three other chickens for each nutritional state.

lysates after immunoprecipitation with α PY20 and immunoblotting with α Shc (Figure 1, lower panel). Surprisingly, in both tissues, only the 52 kDa Shc isoform was revealed. As shown later, other forms of Shc can also be phosphorylated on tyrosine residues. In both tissues, tyrosine phosphorylation of the 52 kDa Shc isoform was reduced in the fasting state compared with the fed or refed states (Figure 1, lower panel). After quantitative analysis using NIH Image densitometry and normalization (100% for the fed state), differences were significant in liver (fasted = 69 ± 11 and refed = 95.5 ± 8.5 ; *n* = 3 per nutritional state) and in muscle (fasted = 59.4 ± 16.9 and refed = 121.8 ± 6.4 ; *n* = 3).

A 594 bp fragment was amplified by RT-PCR using primers flanking the PTB domain of mouse Shc cDNA, and was then cloned and sequenced. The deduced amino acid sequence of this chicken cDNA (not shown; GenBank accession number AF 035949) presents 89% similarity with the mammalian (rat or human) Shc PTB domain sequences, which indicates a high conservation of this gene, at least in the PTB region. Shc mRNA was quantified in liver and muscle, according to the nutritional status, by RT-PCR and Southern blots, using 18 S RNA as an internal control. After normalization (100% for fed state), the level of Shc messenger was not dependent upon the nutritional state in liver (refed = 98.2 ± 2.4 and fasted = 104 ± 6 ; *n* = 3 per nutritional state) and in muscle (refed = 105 ± 10 and fasted = 107 ± 1.4 ; *n* = 3), which fits with the fact that the amount of Shc protein was not altered by the nutritional state (see earlier).

Co-immunoprecipitation of IR and Shc *in vivo* in chicken liver and muscle

Homogenates from chicken liver and muscle were immunoprecipitated with an anti-IR (B10) antibody and immunoblots were assessed using α Shc. Independently of the nutritional state,

Figure 2 Co-immunoprecipitation of the IR and the 52 kDa Shc isoform

(A, B) Liver (lanes 1, 2, 3) and muscle (lanes 4, 5, 6) samples from chickens fed *ad libitum* (lanes 1, 4), refed (lanes 2, 5) and fasted (lanes 3, 6) were solubilized and immunoprecipitated with an IR antibody (B10) and blotted either with α Shc (A) or α PY20 (B). Bands were revealed by ECL. (C) The same samples as in (A) and (B) from liver were immunoprecipitated with α EGF-R and membranes were blotted with α PY20; bands were revealed by ECL. (D) Liver lysates were immunoprecipitated with either anti-IR antibody (B10) or α EGF-R. Membranes were blotted with α Shc and revealed by ECL and using as molecular mass markers the three Shc isoforms from transduction laboratories. Finally, supernatants from the first B10 or α EGF-R immunoprecipitations were further immunoprecipitated with the reciprocal α EGF-R and B10 respectively (inset in the lower panel). Membranes were also blotted with α Shc.

and in both tissues, the 52 kDa and, to a relatively lesser extent, the 46 kDa Shc isoforms, co-immunoprecipitated with IR (Figure 2A). Furthermore, the amount of Shc (52+46 kDa isoforms) associated with the IR was regulated by the nutritional status. Fasting reduced the amount of Shc associated with the IR in liver and muscle by 15 and 20% respectively ($P < 0.05$, compared with the refed state; using data from the fed state as a reference after densitometric analysis with NIH Image software). Immunoprecipitation with a guinea pig or a human non-immune serum did not reveal any form of Shc (results not shown). Thus, IR and Shc do co-immunoprecipitate in two insulin target chicken tissues; furthermore, this association is regulated by the nutritional status. It is worth noting that, in liver, the decrease in the amount of Shc associated with IR induced by fasting is observed, despite an increase in membrane IR number.

To demonstrate whether Shc associated with IR is phosphorylated on tyrosine residues, liver and muscle homogenates were immunoprecipitated with B10 and immunoblotting was assessed with α PY20 (Figure 2B). In both tissues, the three Shc isoforms were phosphorylated on tyrosine residues; the 52 kDa

isoform, which is abundant (Figure 2A), was largely tyrosine phosphorylated (Figure 2B). Furthermore, the level of phosphorylation of 52 (and 46) kDa forms was qualitatively decreased in the fasting state in both tissues ($n = 2$). Refeeding for 30 min restored the phosphorylation of the two isoforms. These alterations fit with those quantified after α PY20 immunoprecipitation (Figure 1, lower panel). Thus, Shc isoforms associated with IR are tyrosine phosphorylated. The same solubilized liver materials were also immunoprecipitated with an α EGF-R: the three (66, 52 and 46 kDa) Shc isoforms were associated with EGF-R and phosphorylated on tyrosine residues. However, the level of phosphorylation of Shc associated with EGF-R did not qualitatively appear to be regulated by the nutritional state (Figure 2C; $n = 2$).

To determine the preferential association of IR with the 52 kDa Shc isoform, liver material from the same solubilization was immunoprecipitated using either IR antibody (B10) or EGF-R antibody (Figure 2D). Blotting with α Shc revealed that IR co-immunoprecipitated, mainly with the 52 kDa isoform, and to a lesser extent with the 46 kDa, whereas EGF-R co-immunoprecip-

Figure 3 Co-immunoprecipitation of IRS-1 and Shc in liver and muscle

Liver (lanes 1, 2) and muscle (lanes 3, 4) samples from fed chicken were solubilized and immunoprecipitated with α IRS-1 and membranes were blotted with α Shc. The bands were revealed by ECL.

itated with the three isoforms (66, 52 and 46 kDa), as already observed (Figure 2C). Thus, *in vivo*, IR interacts mainly with the 52 kDa isoform, whereas EGF-R interacts with the three Shc isoforms. In order to further establish these specificities, supernatants from the first immunoprecipitates were again immunoprecipitated using a reciprocal antibody. The B10 second immunoprecipitate mainly revealed the 52 kDa Shc isoform from the first α EGF-R supernatant, whereas α EGF-R mainly revealed the 46 kDa isoform, and, to a lower extent, the 52 kDa isoform from the first B10 supernatant (Figure 2D, inset). The absence of the 66 kDa Shc isoform in the second α EGF-R immunoprecipitate may be accounted for by a low level of this isoform and/or its dissociation from EGF-R during the first B10 immunoprecipitation.

In vivo association of IRS-1 and Shc in liver and muscle

The possibility that IRS-1 and Shc interact was investigated in liver and muscle from fed chickens. Homogenates were immunoprecipitated with α IRS-1 and immunoblotting was assessed using α Shc. Two bands corresponding to the 52 and 46 kDa Shc isoforms appeared in both liver and muscle (Figure 3). However, in each case, the 46 kDa isoform was more abundant, which contrasts with the results obtained for the co-immunoprecipitation with IR, where the major associated form was the 52 kDa isoform (Figures 2A, 2B and 2D).

Co-immunoprecipitation of Shc with PI 3'-kinase activity and the p85 and p55/p50 isoforms of the regulatory subunit of PI 3'-kinase

In the next experiments, the possibility that Shc and a PI 3'-kinase activity co-immunoprecipitate was investigated in both liver and muscle. First, solubilized materials from liver were immunoprecipitated using either α Shc (Figure 4A) or α PY20 (Figure 4B). In both precipitates and in both tissues, a PI 3'-kinase activity was present. After normalization (100% = fed state), in liver, fasting significantly ($P < 0.05$) decreased the Shc-associated PI 3'-kinase activity, whereas refeeding restored it (Figure 4A). In contrast, in muscle, the associated activity was not altered by nutritional status (Figures 4A and 4B). Whether or not these two antibodies precipitate a unique entity, and therefore the same PI 3'-kinase activity, is examined later.

To identify the PI 3'-kinase regulatory subunits which are associated with Shc, liver and muscle homogenates were immuno-

Figure 4 PI 3'-kinase activity immunoprecipitated with α Shc or α PY20

Liver and muscle samples from fed, refed and fasted chickens were solubilized and immunoprecipitated with either α Shc (A) or α PY20 (B). PI 3'-kinase activity was measured in these immunoprecipitates as described in the Experimental section. γ - 32 P incorporated into phosphatidylinositol was determined using STORM apparatus and expressed as arbitrary units. Within each experiment, data were normalized using PI 3'-kinase activity from fed chickens as a reference. Results are presented as the means $\pm$ S.E.M., $n = 5$. *Indicates significant differences with $P < 0.05$.

precipitated with either α Shc or α PY20 and blotted with α p85. In mammals, this antibody cross-reacts with the three isoforms (p85, p55 and p50). In liver, irrespective of the immunoprecipitating antibody (Figures 5A and 5B, left part), two bands with apparent molecular masses of 85 and 50–55 kDa appeared. After quantitative analysis using NIH Image densitometry and normalization (100% fed state), the association of the p85 subunit with Shc (fasted = 74.5 ± 7.5 , refed = 121.2 ± 8.5) or with proteins containing phosphotyrosine (fasted = 85.1 ± 5.7 , refed = 117.5 ± 4.8) was dependent upon the nutritional state, with a significant increase in the refed state when compared with the fasted state (Figures 5A and 5B; $P < 0.05$, $n = 3$). In muscle, α Shc immunoprecipitated only the p85 subunit (Figure 5A, right

Figure 5 Co-immunoprecipitation of Shc and the regulatory subunits of PI 3'-kinase

(A) Liver (lanes 1, 2, 3) and muscle (lanes 4, 5, 6) samples from chickens fed *ad libitum* (lanes 1, 4), refed (lanes 2, 5) and fasted (lanes 3, 6) were solubilized and immunoprecipitated with α Shc. (B) Muscle (lanes 1, 2, 3) and liver (lanes 4, 5, 6) samples from fasted (lanes 1, 4), refed (lanes 2, 5) and *ad libitum* fed (lanes 3, 6) chickens were immunoprecipitated with α PY20 [note that the order is different from that in (A)]. In (A) and (B), membranes were blotted with α p85. A control band provided by UBI corresponding to the p85 subunit was also included [(A), lane C]. The bands were revealed by ECL and their apparent molecular masses (in kDa) are indicated on the left side of the Figures. (C) Liver and muscle samples from fed chicken were immunoprecipitated with α PY20, α p85 and B10. A normal human serum (HS) was used as control. Membranes were blotted with α Shc. The bands were revealed by ECL. A control containing the three isoforms of Shc from Transduction Laboratories was added (lane C). The apparent molecular masses (in kDa) are indicated on the left side of the Figure.

part) and this association was not dependent upon nutritional state (fasted = 101.8 ± 7.9 , refed = 98.6 ± 5.6). When muscle homogenates were immunoprecipitated with α PY20, both p85 and p55/p50 subunits were revealed (Figure 5B). Again, this association was not altered by the nutritional state (fasted = 113.3 ± 7.2 , refed = 92.9 ± 8.3), which fits with the fact that in α Shc muscle immunoprecipitates PI 3'-kinase activity was not

Figure 6 Co-immunoprecipitation of PI 3'-kinase regulatory subunits with IR, IRS-1 and Shc

Solubilized liver material was submitted to PAGE after either direct application (none) or after immunoprecipitations (α Shc, α IRS-1 or B10). After electrotransfer, membranes were blotted with α p85 and revealed by ECL.

altered by the nutritional state, in contrast with results obtained with liver (see earlier, Figure 4).

The association of Shc with PI 3'-kinase regulatory subunits was further demonstrated using α p85 immunoprecipitation. In immunoprecipitates from liver and muscle, blotting with α Shc revealed only the presence of the 52 kDa Shc isoform (Figure 5C, second lane from the left for each tissue). Figure 5(C) further demonstrates the specificity of Shc interactions: in both tissues, the 52 kDa Shc isoform was the major form of Shc that was immunoprecipitated by α PY20 (as in Figure 1, lower panel) or α IR (B10, as in Figure 2). In contrast, non-immune human serum did not precipitate the 52 kDa Shc isoform (HS, Figure 5C).

Possible simultaneous interaction of IR with Shc and IRS-1

Finally, the possible existence of different subtypes of IR complexes signalling through IRS-1 or Shc was investigated on the basis of PI 3'-kinase activity. This question was addressed only in liver. Solubilized liver material was immunoprecipitated using either α Shc, α IRS-1 or α PY20 and PI 3'-kinase activity was determined in the three immunoprecipitates. The PI 3'-kinase activity, expressed as arbitrary units, associated with each of these immunoprecipitates was very similar: 18.5 ± 2.9 , 15 ± 2.5 and 16.3 ± 2.6 for α Shc, α IRS-1 and α PY20 respectively. Supernatants from the first α Shc or α IRS-1 immunoprecipitates were subjected to a second immunoprecipitation with the reciprocal α Shc or α IRS-1 antibodies. The residual PI 3'-kinase activity found in the two second precipitates was only 8–12 % of that found in the first precipitates (results not shown). Further comparisons were performed using α Shc, α IRS-1 or B10 immunoprecipitations. Again, PI 3'-kinase activity was found to be very similar in all precipitates. The first B10 supernatant was also further immunoprecipitated with either α Shc, α IRS-1 or B10. In all of these second precipitates, residual PI 3'-kinase activity was less than 5 % of the initial activity (results not shown). Finally, samples of first immunoprecipitates (α Shc, B10 and α IRS-1) were also submitted to SDS/PAGE and blotted with α p85. Figure 6 shows that after direct application, and in all immunoprecipitates, the same three bands appeared, with apparent molecular masses of 85, 55 and 50 kDa and about the same amount of each. These three proteins correspond to the three isoforms of the regulatory subunits of the PI 3'-kinase. These results indicate that no distinct pools of PI 3'-kinase activity

associated with either IR, IRS-1 or Shc can be identified. Consequently, we may conclude that both Shc (mainly the 52 kDa Shc isoform) and IRS-1 associate simultaneously with the IR and a similar PI 3'-kinase activity and/or amount of enzyme.

DISCUSSION

Shc, an intracellular substrate and a signalling protein for the tyrosine kinase of IR and various growth factor receptors, is considered to act by initiating the *ras/raf* cascade [6]. We have characterized Shc isoforms in chicken liver and muscle. As in mammalian cells, three isoforms (66, 52 and 46 kDa) were revealed with an antibody directed against mouse Shc. One of the major findings is the demonstration that in both tissues, IR and Shc co-immunoprecipitate, mainly the 52 kDa and to a lesser extent the 46 kDa Shc isoform. Importantly, this association and Shc phosphorylation were dependent upon the nutritional state. These changes are most likely accounted for by changes in plasma insulin levels. Current studies (J. Dupont, M. Derouet, J. Simon and M. Taouis, unpublished work) demonstrate that in chicken liver and muscle, tyrosine phosphorylation of Shc (associated or not with the IR) is enhanced 10 min after intra-peritoneal injection of 1 unit of insulin/kg. Furthermore, the alterations in tyrosine phosphorylation of Shc presently observed mirrored the changes previously observed for liver IR β subunit and IRS-1 phosphorylation [26]. Surprisingly, however, the phosphorylation of muscle IR β subunit and IRS-1 were not found to be clearly altered by these nutritional states [26], in contrast with the present results where the degree of phosphorylation of Shc in muscle was altered by the nutritional state. Whether this observation only reflects a difference in the amount of the two substrates or indicates a different role for the two substrates, particularly in muscle, remains unknown.

To our knowledge, the co-immunoprecipitation of IR and Shc has not been previously demonstrated in untransfected mammalian cells, despite the fact that an insulin-dependent tyrosine phosphorylation of Shc has been observed [8]. An interaction between the two molecules has been suggested by the use of a very sensitive technique, the two hybrid system, in which IR and Shc are overexpressed [17,19,20]. In normal mammalian cells, Shc largely associates with EGF-R [20]. In chicken muscle and liver, the 52 kDa, and to a lesser extent the 46 kDa, Shc isoforms were found to associate with IR, whereas the three Shc isoforms were able to associate with EGF-R. Whether this is a consequence of differences in relative affinity or relative abundance of the different forms remains unclear.

To date, Shc has been considered to be independent of the PI 3'-kinase pathway in insulin and growth factor receptor signalling. Nevertheless, one report [30] has indicated the possible interaction between Shc and the p85 regulatory subunit of PI 3'-kinase in BaF3 cells expressing p20BCR/abl (chronic myelogenous leukaemia cells). The authors [30] concluded that this interaction is independent of Shc tyrosine phosphorylation and involves the proline-rich domain of Shc and the SH3 domain of the p85 subunit [30,31]. Here, we show that, at least in liver, PI 3'-kinase activity is associated with a large complex which involves: IR-IRS-1-Shc. This complex is regulated by nutritional status. The three isoforms of the PI 3'-kinase regulatory subunits (p50, p55 and p85), which in mammals are coded by the *p85 α* gene [32], are present in the whole complex. In muscle, Shc co-immunoprecipitated only with the p85 isoform, even though the three regulatory subunits of PI 3'-kinase were identified in this tissue after α PY20 immunoprecipitation. The PI 3'-kinase regulatory subunits appear to interact more specifically with the

52 kDa Shc isoform in both tissues. Taken together, these results suggest that the expression and the role of the three isoforms of the regulatory subunits of PI 3'-kinase may be tissue specific, supporting previous observations made by others [32]. The specific association of the p50 isoform with the 52 kDa Shc isoform in liver may, at least partly, explain the sensitive regulation of PI 3'-kinase activity which has been found in this tissue in response to alterations of the nutritional state and the subsequent variation in plasma insulin levels. As previously shown in rats, the p50 isoform is highly expressed in liver and is markedly responsive to insulin [32].

Two additional results strongly suggest the existence of another complex involving IRS-1 and the 46 kDa Shc isoform, but no PI 3'-kinase activity in liver and muscle. Using a systemic peptide strategy, a direct binding between IRS-1 and Shc has been suggested to be possible through the SH2 domain of Shc and Tyr-690 of IRS-1, although with weak affinity [33].

In conclusion, Shc (mainly the 52 kDa isoform) interacts with the IR. This association and Shc tyrosine phosphorylation in chicken muscle and liver are dependent upon nutritional status. A quaternary complex, which includes IR, the 52 kDa Shc isoform, IRS-1 and PI 3'-kinase regulatory subunits and PI 3'-kinase activity, has been demonstrated. In muscle, only the p85 isoform takes part in the large complex. Another complex, including IRS-1 and the 46 kDa Shc, but no PI 3'-kinase activity, is also present. The present demonstration that Shc associates with the IR in chicken tissues strongly suggests that this phenomenon also exists in mammalian tissues. Several questions remain to be answered: why is this complex so abundant in chicken tissues, is it accounted for by a difference in the relative abundance of Shc compared with IRS-1 and does this change insulin signalling in this species?

This work was supported financially by the Association Française des Myopathies.

REFERENCES

- 1 Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) *Nature (London)* **352**, 73–77
- 2 Sun, X. J., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G., Glasheen, E., Lane, W. S., Pierce, J. H. and White, M. F. (1995) *Nature (London)* **377**, 173–177
- 3 Patti, M. E., Sun, X. J., Bruening, J. C., Araki, E., Lipes, M. A., White, M. F. and Kahn, C. R. (1995) *J. Biol. Chem.* **270**, 24670–24673
- 4 Lavan, B. E., Lane, W. S. and Lienhard, G. E. (1997) *J. Biol. Chem.* **272**, 11439–11443
- 5 Lavan, B. E., Fantin, V. R., Chang, E. T., Lane, W. S., Keller, S. R. and Lienhard, G. E. (1997) *J. Biol. Chem.* **272**, 21403–21407
- 6 Pellicci, G., Lanfranccone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Pawson, T. and Pellicci, P. G. (1992) *Cell* **70**, 93–104
- 7 Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C. H. and Claesson-Welsh, L. (1994) *J. Biol. Chem.* **269**, 15337–15343
- 8 Borello, M. G., Pellicci, G., Arighi, E., De Filippis, L., Greco, A., Bongarzone, I., Rizzetti, M. G., Pellicci, P. G. and Pierotti, M. (1994) *Oncogene* **9**, 1661–1668
- 9 Sasaoka, T., Rose, D. W., Jhun, B. H., Saltiel, A. R., Draznin, B. and Olefsky, J. M. (1994) *J. Biol. Chem.* **269**, 13689–13694
- 10 Migliaccio, E., Mele, S., Salcini, A. E., Pellicci, G., Venus Lai, K. M., Superti-Furga, G., Pawson, T., Difiore, P. P., Lanfranccone, L. and Pellicci, P. G. (1997) *EMBO J.* **16**, 706–716
- 11 Pronk, G. J., McGlade, J., Pellicci, G., Pawson, T. and Bos, J. L. (1993) *J. Biol. Chem.* **268**, 5748–5753
- 12 Saltiel, A. R. (1996) *Am. J. Physiol.* **270**, E375–E385
- 13 White, M. F. and Kahn, C. R. (1994) *J. Biol. Chem.* **269**, 1–4
- 14 Pellicci, G., Dente, L., De Giuseppe, A., Verducci-Galletti, B., Giuli, S., Mele, S., Vetriani, C., Giorgio, M., Pandolfi, P. P., Cesareni, G. and Pellicci, P. G. (1996) *Oncogene* **13**, 633–641
- 15 Bonfini, L., Migliaccio, E., Pellicci, G., Lanfranccone, L. and Pellicci, P. G. (1996) *Trends Biochem. Sci.* **21**, 257–261

- 16 Harun, R. B., Smith, K. K., Leik, J. P., Markham, A. F., Norris, A. and Morrison, J. F. (1997) *Genomics* **42**, 349–352
- 17 Okada, S., Yamauchi, K. and Pessin, J. E. (1995) *J. Biol. Chem.* **270**, 20737–20741
- 18 Giorgetti, S., Pelicci, P. G., Pelicci, G. and Van Obberghen, E. (1994) *Eur. J. Biochem.* **223**, 195–202
- 19 Gustafson, T. A., He, W., Craparo, A., Schaub, C. D. and O'Neill, T. J. (1995) *Mol. Cell. Biol.* **15**, 2500–2508
- 20 He, W., Thomas, T. J. and Gustafson, T. A. (1995) *J. Biol. Chem.* **270**, 23258–23262
- 21 Kasus-Jacobi, A., Perdureau, D., Tartare-Deckert, S., Van Obberghen E., Girard J. and Burnol, A. F. (1997) *J. Biol. Chem.* **272**, 17166–17170
- 22 Yonezawa, K., Ando, A., Kaburagi, Y., Yamamoto-Honda, R., Kitamura, T., Hara, K., Nakafuku, M., Okabayashi, Y., Kadowaki, T., Kaziro, Y. and Kasuga, M. (1994) *J. Biol. Chem.* **269**, 4634–4640
- 23 Kovacina, K. S. and Roth, R. A. (1993) *Biochem. Biophys. Res. Commun.* **192**, 1303–1311
- 24 Taouis, M., Taylor, S. I. and Reitman, M. (1996) *Gene* **178**, 51–55
- 25 Taouis, M., Dupont, J., Gillet, A., Derouet, M. and Simon, J. (1998) *Mol. Cell. Endocrinol.* (1998) **137**, 177–186
- 26 Dupont J., Derouet M., Simon, J. and Taouis, M. (1998) *Am. J. Physiol.* **274**, E309–E316
- 27 Taouis, M., Levy-Toledano, R., Roach, P., Taylor, S. I. and Gorden, P. (1994) *J. Biol. Chem.* **269**, 14912–14918
- 28 Taouis, M., Levy-Toledano, R., Roach, P., Taylor, S. I. and Gorden, P. (1994) *J. Biol. Chem.* **269**, 27762–27766
- 29 Levy-Toledano, R., Taouis, M., Blaettler, D., Taylor, S. I. and Gorden, P. (1994) *J. Biol. Chem.* **269**, 31178–31182
- 30 Harrison-Findik, D., Susa, M. and Varticovski, L. (1995) *Oncogene* **10**, 1385–1391
- 31 Ren, R., Mayer, B. J., Ciccetti, P. and Baltimore, D. (1993) *Science* **259**, 1157–1161
- 32 Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushima, Y., Hosaka, T., Suzuki, M., Shin, B. C. et al. (1997) *J. Biol. Chem.* **272**, 7873–7882
- 33 Ward, C.W., Gough, K. H., Rashe, M., Wan, S. S., Tribbick, G. and Wang, J. (1996) *J. Biol. Chem.* **271**, 5603–5609

Received 30 April 1998/9 July 1998; accepted 4 August 1998