Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes

Vered RIBON*† and Alan R. SALTIEL*†‡

*Department of Physiology, University of Michigan School of Medicine, Ann Arbor, Michigan 48109, U.S.A., and ‡Department of Cell Biology, Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, Ann Arbor, Michigan 48105, U.S.A.

We report here that the product of the c-Cbl proto-oncogene is prominently tyrosine phosphorylated in response to insulin in 3T3-L1 adipocytes. The tyrosine phosphorylation of c-Cbl reaches a maximum within 1–2 min after stimulation by insulin and gradually declines thereafter. The tyrosine phosphorylation of c-Cbl was also observed after treatment of 3T3-L1 adipocytes with epidermal growth factor, whereas platelet-derived growth factor had no effect. After insulin-dependent tyrosine phosphorylation, c-Cbl specifically associates with fusion proteins containing the Src homology 2 (SH2) domains of Crk and the Fyn tyrosine kinase, but not with fusion proteins containing the SH2 domains of either the p85 subunit of phosphatidylinositol 3′-kinase or the tyrosine phosphatase SHPTP2/Syp. Furthermore insulin stimulates the association of c-Cbl with endogenous c-Crk and Fyn in intact 3T3-L1 adipocytes. The tyrosine phosphorylation of c-Cbl is regulated during adipocyte differentiation. Neither insulin-like growth factor 1 nor insulin stimulated the tyrosine phosphorylation of c-Cbl in 3T3-L1 fibroblasts. Moreover, c-Cbl is not tyrosine phosphorylated in response to insulin in cells expressing high levels of the human insulin receptor, or in hepatocytes, despite comparable levels of c-Cbl expression. These results suggest that c-Cbl might have a novel function in the regulation of insulin receptor intracellular signalling in 3T3-L1 adipocytes.

INTRODUCTION

Although the intracellular events that mediate insulin action are not fully understood, the regulation of protein tyrosine phosphorylation has a critical role [1]. On insulin binding, the insulin receptor undergoes autophosphorylation on tyrosine residues, resulting in increased kinase activity and the tyrosine phosphorylation of intracellular substrates [2,3]. Unlike other receptor tyrosine kinases such as those for epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), the tyrosine-phosphorylated insulin receptor does not associate strongly with downstream Src homology 2 (SH2) domain-containing signalling molecules, but instead utilizes a family of docking proteins that include the insulin receptor substrate 1 (IRS-1), IRS-2 and the adapter protein Shc [2,4–10]. The activated insulin receptor rapidly phosphorylates IRS-1 on multiple tyrosine residues to generate binding sites for SH2-containing proteins, including the adapter proteins Grb2 and Nck [11,12], the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI 3′K) [13,14], the tyrosine phosphatase SHPTP2/Syp [15], and more recently Fyn [16]. Binding of PI 3′K to phosphorylated IRS-1 leads to activation of the lipid kinase in response to insulin [13]. Phosphorylated Shc is thought to link insulin receptor signalling to Ras activation by binding to the Grb2–Sos complex [5,6,12,17,18]. Recently, a novel Grb2-associating protein (GAB1) was cloned that is similar to IRS-1 and binds several SH2-containing proteins after stimulation by insulin [19]. Although activation of these known pathways is clearly important in insulin signal transduction [4,10,12,13,20], recent studies indicated that stimulation of neither mitogen-activated protein kinase nor PI 3′K by insulin is sufficient for the stimulation of glucose transport, lipid synthesis or glycogen synthesis in 3T3-L1 adipocytes, suggesting that additional signalling pathways exist [21,22].

The c-Cbl proto-oncogene product is the 120 kDa cellular homologue of the v-*cbl* oncogene, which is transforming in early B-lineage and myeloid cells [23,24]. c-Cbl resembles a DNA-binding transcription factor, with a nuclear localization sequence, a zinc finger-like motif and a leucine zipper. However, it is localized predominately in the cytoplasm, whereas the truncated protein encoded by v-*cbl* is localized in both the cytoplasm and the nucleus, where it can bind DNA [23–26]. The sequence of c-Cbl also predicts a number of consensus tyrosine phosphorylation sites and a proline-rich SH3-binding domain. c-Cbl becomes tyrosine phosphorylated after activation of a variety of tyrosine-kinase signalling pathways, and after transformation by v-Abl or Bcr–Abl [26–30]. Tyrosine-phosphorylated c-Cbl in activated T-cells can bind to the SH2 domains of Fyn, Lck [27,31], the p85 subunit of PI 3′K [32] and c-Crk [33]. Moreover, the association of c-Crk with tyrosine-phosphorylated c-Cbl correlates well with cellular transformation in Bcr-Abl or mutant, oncogenic c-Cbl (70Z) expressing cells [33]. Additionally, c-Cbl binds to the SH3 domains of the adapters Grb2 and Nck [27,29,34], and the Fyn and Lck tyrosine kinases independently of cell stimulation [31].

In this study we show that c-Cbl is rapidly tyrosine phosphorylated in response to insulin in differentiated 3T3-L1 adipocytes. On phosphorylation, c-Cbl forms insulin-dependent complexes with endogenous c-Crk and Fyn. These results suggest a role for tyrosine-phosphorylated c-Cbl in insulin action in the metabolically responsive 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials

Insulin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), EGF from Becton Dickinson (Bedford, MA, U.S.A.), PDGF from R&D Systems, (Minneapolis, MN, U.S.A.) and insulin-like growth factor 1 (IGF-1) from Calbiochem Novabiochem Corp.

Abbreviations used: CHO/IR, Chinese hamster ovary cells expressing human insulin receptors; EGF, epidermal growth factor; GST, glutathione S-transferase; HIRC, Rat1 fibroblasts transfected with the human insulin receptor; IGF, insulin-like growth factor; IRS-1, insulin receptor substrate 1; PDGF, platelet-derived growth factor; PI 3′K, phosphatidylinositol 3′-kinase; SH2, Src homology 2.

‡ To whom correspondence should be addressed at Parke-Davis.

(San Diego, CA, U.S.A.). The polyclonal anti-c-Cbl and anti-Fyn antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(insulin receptor) antibodies were from Oncogene Science (Cambridge, MA, U.S.A.). Anti-Shc, anti-Crk and the anti-phosphotyrosine (RC20H) antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). The polyclonal anti-IRS-1 antisera were developed as described [35]. The anti-phosphotyrosine 4G10 antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Protein G/protein A–agarose were from Oncogene Science. Glutathione–Sepharose was purchased from Pharmacia Biotech (Piscataway, NJ, U.S.A.). The enhanced chemiluminescence (ECL) system was from Amersham Life Science (Arlington Heights, IL, U.S.A.).

Cells and culture conditions

3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (4.5 g/l glucose) supplemented with 10 % (v/v) calf serum, 1 % (w/v) penicillin and 1 % (w/v) streptomycin. Differentiation to adipocytes was induced as described [36] and experiments were conducted 2–4 days after induction of differentiation. Chinese hamster ovary cells expressing 3×10^6 human insulin receptors per cell (CHO/IR) were a gift from Dr. J. Pessin [37]. Rat1 fibroblasts transfected with the human insulin receptor (HIRC) were a gift from Dr. J. Olefsky [38]. Rat hepatoma KRC-7 cells were kindly provided by Dr. J. Koontz (University of Tennessee, Knoxville, TN, U.S.A.) and maintained in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum, 1 % (w/v) penicillin and 1 % (w/v) streptomycin. The cells grown in 10 cm dishes were serum-deprived for 12–18 h before hormonal treatment. Unless otherwise indicated, 100 nM insulin, 100 ng/ml EGF, 100 ng/ml PDGF or 100 ng/ml IGF-1 were added directly to the medium and the incubation continued for the indicated periods at 37 °C.

Immunoprecipitations and immunoblotting

After hormonal treatment, cells were washed twice with ice-cold PBS and incubated for 15 min on ice in lysis buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.5 % Triton X-100/1 mM EDTA/1 mM sodium orthovanadate/10 mM NaF/10 μg/ml aprotinin/10 μg/ml leupeptin/1 mM PMSF]. The lysates were clarified by centrifugation at 10000 ***g*** for 15 min at 4 °C. Equal protein concentrations of lysates were immunoprecipitated with the indicated antibodies. After 4 h at 4 °C, immunoprecipitates were mixed with protein G/protein A–agarose for 1 h and the immunocomplexes were washed four times with lysis buffer and solubilized at 100 °C for 5 min in 25 μl of SDS gel sample buffer. Immunoprecipitated proteins were resolved by SDS/PAGE and transferred to nitrocellulose membranes. Individual proteins were detected with the specified antibodies, and phosphotyrosine was detected with a mixture of the anti-phosphotyrosine antibodies. Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies followed by the enhanced chemiluminescence system. To reprobe immunoblots, the nitrocellulose membranes were incubated for 30 min at 60 °C with 62.5 mM Tris/HCl, pH 6.8, containing 2 % SDS and 0.7 % 2-mercaptoethanol and then were washed extensively with 10 mM Tris/HCl (pH 8)/150 mM NaCl.

Binding assays *in vitro*

The glutathione S-transferase (GST) fusion protein containing the SH2 domain of Crk was a gift from Dr. R. B. Birge and Dr. H. Hanafusa [39]. The generation and production of the GST fusion proteins containing the C-terminal SH2 domain of the p85 regulatory subunit of PI 3′K, both SH2 domains of Syp or the SH2 domain of Fyn were as described elsewhere [40–42]. The fusion proteins were purified on glutathione–Sepharose beads, quantified against a BSA standard, and analysed on SDS/PAGE by staining with Coomassie Blue (purity was more than 95 %). For association experiments *in vitro*, lysates from 5×10^6 cells in lysis buffer were incubated with 2.5–5 μg of GST fusion proteins bound to glutathione–Sepharose beads for 90 min at 4 °C. After the beads had been washed three times with lysis buffer, the adsorbed proteins were eluted and analysed as described above for the immunoprecipitates.

RESULTS

Insulin stimulates tyrosine phosphorylation of c-Cbl in 3T3-L1 adipocytes

On differentiation into adipocytes, 3T3-L1 cells respond to insulin with a large increase in glucose transport, lipid and glycogen synthesis [36]. To explore a possible role for c-Cbl in insulin action, we examined the tyrosine phosphorylation of this protein in 3T3-L1 adipocytes. Lysates were prepared from untreated or insulin-stimulated 3T3-L1 adipocytes and incubated with anti-c-Cbl antibodies. The resulting immunoprecipitates were separated by SDS/PAGE and tyrosine phosphorylation was analysed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Figure 1(A), c-Cbl was not tyrosine phosphorylated in unstimulated 3T3-L1 cells. The addition of insulin caused the marked tyrosine phosphorylation of the 120 kDa c-Cbl protein. c-Cbl phosphorylation reached maximal levels as early as 1 min after stimulation by insulin and declined thereafter, with substantial phosphorylation still detectable for at least 30 min (Figure 1A, anti-pY blot). The blot was completely stripped of the anti-phosphotyrosine antibodies and reprobed with anti-c-Cbl antibodies (Figure 1A, anti-c-Cbl blot). c-Cbl was precipitated equally from all samples and co-migrated with the phosphotyrosine-containing protein detected in this size range. Similarly to the insulin-dependent phosphorylation of c-Cbl, the tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 also reached maximal levels at 1 min and declined by 5 min as observed in 3T3-L1 adipocytes lysates (Figure 1B). Thus c-Cbl is a major, early substrate of tyrosine phosphorylation after the stimulation of 3T3-L1 adipocytes by insulin.

3T3-L1 adipocytes also express significant numbers of receptors for other growth factors such as EGF and PDGF. We next investigated whether c-Cbl is phosphorylated in response to EGF or PDGF in these cells. c-Cbl was immunoprecipitated from cell lysates of unstimulated and hormone-stimulated 3T3-L1 adipocytes and immunoblotted with anti-phosphotyrosine antibodies. As shown in Figure 1(C) (anti-pY blot), treatment of 3T3-L1 adipocytes with EGF (100 ng/ml) produced the tyrosine phosphorylation of c-Cbl, in agreement with previous results reported for fibroblasts overexpressing the EGF receptor [28]. Interestingly, treatment of these cells with PDGF (100 ng/ml) had no effect on the tyrosine phosphorylation of c-Cbl. The amounts of c-Cbl immunoprecipitated were identical in the untreated and hormone-stimulated cells (Figure 1C, anti-c-Cbl blot).

To characterize further the insulin-dependent tyrosine phosphorylation of c-Cbl, we stimulated 3T3-L1 adipocytes with different concentrations of insulin. After treatment with insulin for 1 min, lysates were prepared and immunoprecipitated with anti c-Cbl antibodies. As shown in Figure 2, the anti-phosphotyrosine immunoblotting revealed that phosphorylation of c-Cbl

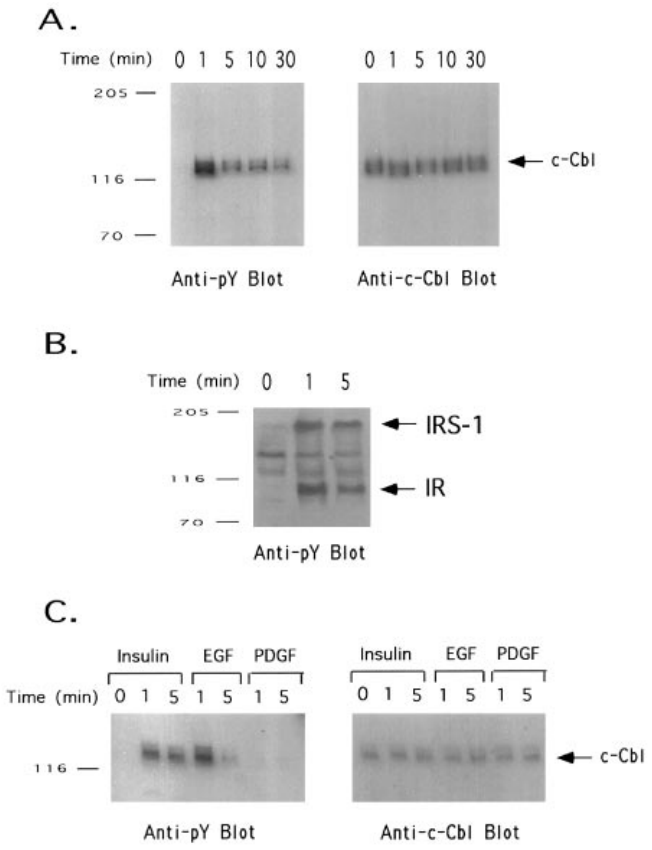

Figure 1 Insulin stimulates the tyrosine phosphorylation of c-Cbl in 3T3-L1 adipocytes

(**A**) Serum-starved 3T3-L1 adipocytes were stimulated with insulin (100 nM) for the indicated times at 37 °C and the cell lysates were immunoprecipitated with anti-c-Cbl antibodies. The immunoprecipitates were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies (anti-pY blot) and then reprobed with anti-c-Cbl antibodies (anti-c-Cbl blot). (**B**) Cell lysates (30 μg per lane) prepared from 3T3-L1 adipocytes treated with insulin (100 nM) for the indicated periods were directly analysed by immunoblotting with anti-phosphotyrosine antibodies (anti-pY blot). (**C**) Cell lysates prepared from 3T3-L1 adipocytes treated with insulin (100 nM), EGF (100 ng/ml) or PDGF (100 ng/ml) for the indicated periods were immunoprecipitated with anti-c-Cbl antibodies followed by immunoblotting with anti-phosphotyrosine antibodies (anti-pY blot). The blots were stripped of antibodies and reprobed with anti-c-Cbl antibodies (anti-c-Cbl blot). Shown are representative results obtained from five separate experiments. The positions of molecular mass markers (in kDa) are indicated at the left. The arrows indicate the positions of c-Cbl, IRS-1 and the insulin receptor (IR).

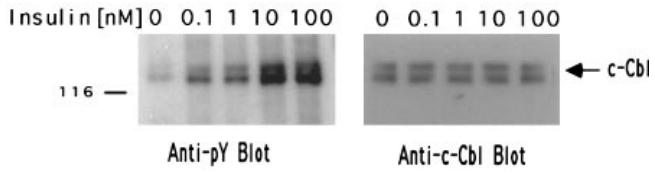

Figure 2 Insulin dose response of c-Cbl tyrosine phosphorylation in 3T3-L1 adipocytes

Serum-starved 3T3-L1 adipocytes were incubated with 0–100 nM insulin for 1 min at 37 °C as indicated. Cell lysates were immunoprecipitated with anti-c-Cbl antibodies and samples were analysed by immunoblotting with anti-phosphotyrosine antibodies (anti-pY blot). The same blot was stripped of antibodies and reprobed with anti-c-Cbl antibodies (anti-c-Cbl blot). The position of a 116 kDa molecular mass marker is indicated at the left.

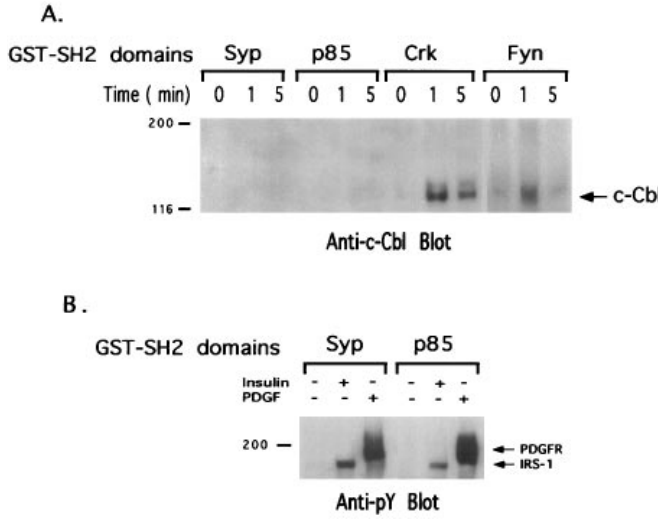

Figure 3 Preferential binding of GST–Crk-SH2 and GST–Fyn-SH2 to c-Cbl from insulin-stimulated 3T3-L1 adipocytes

(**A**) 3T3-L1 adipocytes were stimulated with insulin (100 nM) for the indicated periods at 37 °C. GST–Syp-SH2, GST–p85-SH2, GST–Crk-SH2 or GST–Fyn-SH2 fusion proteins immobilized on glutathione–agarose beads were incubated with cell lysates for 90 min at 4 °C. Bound proteins were eluted and separated by SDS/PAGE followed by immunoblotting with anti-c-Cbl antibodies. The arrow indicates the position of c-Cbl. (**B**) Cell lysates from 3T3-L1 adipocytes stimulated with insulin (100 nM) or PDGF (100 ng/ml) for 1 min at 37 °C, or left untreated, were incubated with GST–Syp-SH2 or GST–p85-SH2 fusion proteins as described above. The bound proteins were immunoblotted with anti-phosphotyrosine antibodies. Shown are representative results obtained from three separate experiments. The arrows indicate the positions of IRS-1 and the PDGF receptor (PDGFR). The positions of molecular mass markers (in kDa) are indicated at the left.

was detectable after treatment of cells with as little as 0.1 nM insulin and continued to increase up to 100 nM insulin (Figure 2, anti-pY blot). c-Cbl was precipitated equally from all samples (Figure 2, anti-c-Cbl blot).

Insulin-stimulated tyrosine phosphorylation of c-Cbl induces its association with the SH2 domains of c-Crk and Fyn

Tyrosine-phosphorylated c-Cbl in activated T-cells binds the SH2 domains of Fyn, Lck, the p85 subunit of PI 3′K [27,31,32] and c-Crk [33]. Because tyrosine-phosphorylated IRS-1 is a major binding protein for SH2-containing signalling proteins folllowing stimulation by insulin, we evaluated whether tyrosine-phosphorylated c-Cbl might serve a similar function. 3T3-L1 adipocytes were treated with insulin, and lysates were incubated with GST fusion proteins containing the Crk-SH2 domain, the Fyn-SH2 domain, both SH2 domains of Syp and the C-terminal SH2 domain of p85 immobilized on glutathione–agarose beads. The bound proteins were separated by SDS/PAGE and analysed by immunoblotting with anti-c-Cbl antibodies. As shown in Figure 3(A), treatment of cells with insulin induced the association of c-Cbl with the GST–Crk-SH2 fusion protein and to a smaller extent with the GST–Fyn-SH2 domain. The amount of c-Cbl associated with GST–Fyn-SH2 is comparable to that observed in activated Jurkat T cells [27,31]. These interactions were specific, as we could not demonstrate a similar association of c-Cbl with the SH2 domains of Syp or p85. However, Figure 3(B) clearly shows that GST–Syp-SH2 or GST–p85-SH2 fusion proteins precipitated insulin-stimulated tyrosine-phosphorylated IRS-1 and the tyrosine-phosphorylated PDGF receptor from

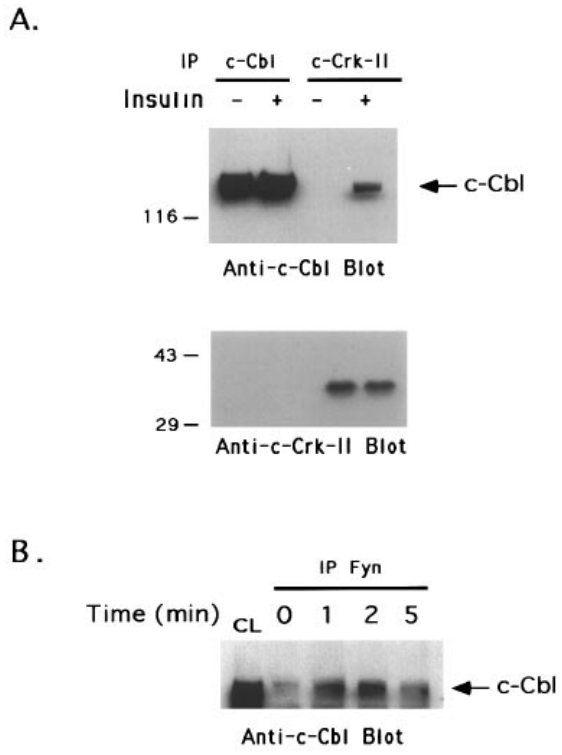

Figure 4 Insulin stimulates the associations of c-Cbl with c-Crk and Fyn in intact 3T3-L1 adipocytes

(**A**) Serum-starved 3T3-L1 adipocytes were stimulated with insulin (100 nM) for 1 min at 37 °C and the cell lysates were immunoprecipitated with anti-c-Crk-II antibodies. After SDS/PAGE and transfer to nitrocellulose, samples were analysed by immunoblotting with c-Cbl antibodies (anti-c-Cbl blot). The blots were stripped of antibodies and reprobed with anti-c-Crk-II antibodies (anti-c-Crk blot). The positions of molecular mass markers (in kDa) are indicated at the left. (**B**) Serum-starved 3T3-L1 adipocytes were stimulated with insulin (100 nM) for the indicated periods at 37 °C. The cell lysates were immunoprecipitated with anti-Fyn antibodies followed by immunoblotting with c-Cbl antibodies. Abbreviations: CL, whole cell lysates; IP, immunoprecipitation.

3T3-L1 adipocytes, as found in previous studies [13–15,43]. We could not detect the co-immunoprecipitation of c-Cbl with p85 in basal or insulin-stimulated cells. These results suggest that c-Cbl has no role in activating PI 3′K in response to insulin in 3T3-L1 adipocytes and that in these cells PI 3′K activity is mainly associated with IRS-1. Similarly, the association of c-Cbl with p85 *in vivo* was not detected in EGF-treated NIH-3T3 cells overexpressing the EGF receptor [28]. In PC-12 cells, EGF, but not NGF or insulin, promoted the association of PI 3′K with c-Cbl [44].

Insulin induces the association of c-Cbl with c-Crk and Fyn *in vivo*

We have shown recently that c-Cbl is the major c-Crk-associated tyrosine-phosphorylated protein in both Bcr-Abl and mutant, oncogenic c-Cbl expressing, cells [33]. The association of c-Cbl with c-Crk and Fyn *in vivo* has been demonstrated following the stimulation of T-cell antigen receptors [31,33]. To elucidate the role of c-Cbl in insulin signalling further, we sought to determine whether c-Cbl would form stable complexes *in vivo* with c-Crk and Fyn. Lysates of unstimulated or insulin-stimulated 3T3-L1 adipocytes were immunoprecipitated with anti-c-Cbl or anti-c-Crk antibodies. As shown in Figure 4(A) (anti-c-Cbl blot), c-Crk does not associate with c-Cbl in unstimulated cells. Insulin rapidly stimulated the association of c-Cbl with c-Crk. Anti-c-Crk immunoblotting revealed that equal amounts of c-Crk were immunoprecipitated from unstimulated or insulin-stimulated 3T3-L1 adipocytes (Figure 4A, anti-c-Crk blot).

We next examined the ability of c-Cbl to form complexes with endogenous Fyn in 3T3-L1 adipocytes (Figure 4B). In unstimulated adipocytes, a low but detectable level of c-Cbl co-immunoprecipitated with Fyn, probably due to an SH3 domain-mediated interaction. Stimulation by insulin increased the amount of c-Cbl associated with endogenous Fyn, reaching maximum levels within 2 min after stimulation by insulin and declining thereafter to basal levels. The enhancement of c-Cbl–Fyn interaction after stimulation by insulin suggests that both the SH2 domain (Figure 3A) and the SH3 domain (results not shown) of Fyn can bind to c-Cbl. Under the conditions of the experiment, the Fyn co-migrated with the IgG heavy chain; therefore we could not directly assay the amount of Fyn immunoprecipitated. However, analysis of the supernatants indicated that Fyn was depleted equally from all samples (results not shown).

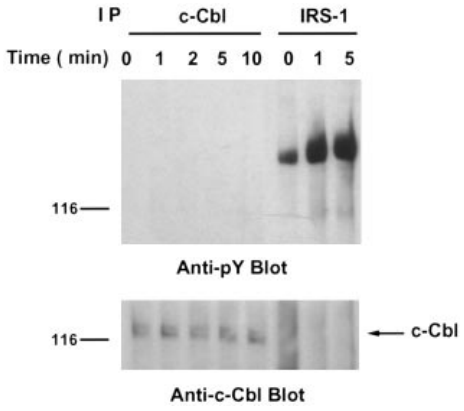

Figure 5 IGF-1 fails to stimulate the tyrosine phosphorylation of c-Cbl in 3T3-L1 fibroblasts

Cell lysates were prepared from 3T3-L1 fibroblasts stimulated with IGF-1 (100 ng/ml) for the indicated periods, incubated with anti-c-Cbl or IRS-1 antibodies and analysed by immunoblotting with anti-phosphotyrosine antibodies (anti-pY blot). The blots were then stripped and re-probed with anti-c-Cbl antibodies (anti-c-Cbl blot). Abbreviation: IP, immunoprecipitation.

IGF-1 failed to induce the tyrosine phosphorylation of c-Cbl in 3T3-L1 fibroblasts

The insulin and IGF-1 receptors share a high degree of similarity in protein sequence and substrate specificity [45]. Because 3T3-L1 fibroblasts express comparable receptors for IGF-1 and insulin (results not shown), it was of interest to determine whether IGF-1 could also induce the tyrosine phosphorylation of c-Cbl in these cells. 3T3-L1 fibroblasts were treated with 100 ng/ml IGF-1 or left untreated. Cell lysates were incubated with anti-c-Cbl or anti-IRS-1 antibodies followed by immunoblotting with anti-phosphotyrosine antibodies (Figure 5). IGF-1 caused a significant tyrosine phosphorylation of IRS-1, which is a common element in both IGF-1 signalling and insulin signalling [45]. Interestingly, treatment with IGF-1 did not stimulate the tyrosine phosphorylation of c-Cbl (Figure 5, anti-pY blot). c-Cbl was precipitated equally from all samples, and comparable amounts of c-Cbl were expressed in 3T3-L1 fibroblasts and adipocytes (Figures 1 and 5, anti-c-Cbl blot). In a similar manner, tyrosine phosphorylation of c-Cbl was not detected after treatment of 3T3-L1 fibroblasts with insulin (results not shown).

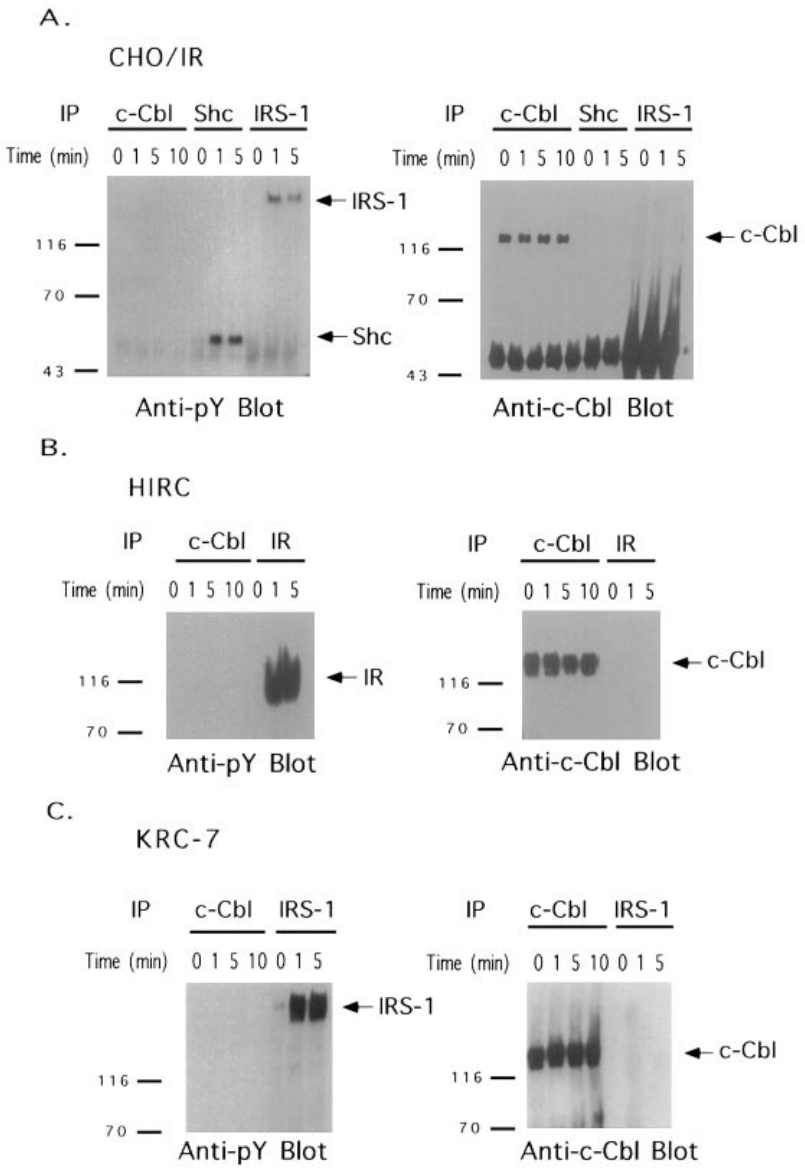

Figure 6 c-Cbl is not tyrosine phosphorylated in response to insulin in CHO/IR cells, HIRC fibroblasts or KRC-7 hepatocytes

CHO/IR cells (**A**), HIRC fibroblasts (**B**) or KRC-7 hepatoma cells (**C**) were stimulated with insulin (100 nM) for the indicated times. Cell lysates were prepared and immunoprecipitated with anti-c-Cbl, anti-Shc, anti-IRS-1 or anti-(insulin receptor) (IR) antibodies. The resulting immunoprecipitates were then subjected to immunoblotting with anti-phosphotyrosine antibodies (anti-pY blot) and then reprobed with anti-c-Cbl antibodies (anti-c-Cbl blot). The positions of molecular mass markers (in kDa) are indicated at the left. The arrows indicate the positions of c-Cbl, IRS-1, Shc and the insulin receptor. Abbreviation: IP, immunoprecipitation.

On differentiation into adipocytes, there is a co-ordinated increase in insulin receptor expression and a decrease in IGF-1 receptor expression [36]. To examine whether the lack of c-Cbl phosphorylation in the fibroblasts is due to a decrease in the number of insulin receptors in the fibroblasts relative to adipocytes, we examined the tyrosine phosphorylation of c-Cbl in cells overexpressing the insulin receptor. CHO cells (CHO/IR) or Rat1 fibroblasts (HIRC) expressing high levels of the insulin receptor, or rat KRC-7 hepatoma cells, were stimulated with 100 nM insulin. c-Cbl, Shc, IRS-1 or the insulin receptor were then immunoprecipitated from cell lysates, followed by immunoblotting with anti-phosphotyrosine or anti-c-Cbl antibodies. As shown in Figure 6 (anti-pY blot), incubation of all of these cell lines with insulin resulted in the tyrosine phosphorylation of known substrates of the insulin receptor, including Shc, IRS-1 and the insulin receptor itself [4,14,37]. Interestingly, in contrast with observations in 3T3-L1 adipocytes, c-Cbl did not undergo insulin-dependent tyrosine phosphorylation in any of these cell lines, although all expressed comparable levels of c-Cbl protein (Figure 6, anti-c-Cbl blot).

DISCUSSION

In contrast with many other receptor tyrosine kinases, including those for EGF or PDGF whose autophosphorylation sites bind directly to SH2 domain-containing signalling proteins, the insulin receptor is believed to transduce signals by the phosphorylation of docking or adapter proteins such as IRS-1, IRS-2, GAB1 and

Shc [2,4–10,19]. Although these tyrosine phosphorylations can lead to the activation of PI 3′K and the mitogen-activated protein kinase cascade in response to insulin, numerous studies have shown that these events are not sufficient to mediate the metabolic effects of insulin [21,22]. These results suggest that there might be additional pathways that are initiated by the tyrosine phosphorylation of other substrates of the insulin receptor. One potential candidate for such a substrate is the c-Cbl protein. The c-*cbl* gene was originally identified as the cellular homologue of a transforming retrovirus that arose in a mouse pre-B-cell lymphoma [23–25]. Although the biochemical and physiological function of c-Cbl remains unclear, this protein is heavily phosphorylated in cells expressing v-Abl or Abl–Bcr tyrosine kinase [26], and is rapidly phosphorylated on tyrosine residues after the activation of a variety of tyrosine kinase signalling pathways in haemopoietic cells [27,29,30], and in response to EGF in cells overexpressing the EGF receptor and in PC-12 cells [28,44].

We report here that c-Cbl is markedly and rapidly tyrosine-phosphorylated in 3T3-L1 adipocytes in response to insulin. Moreover, tyrosine-phosphorylated c-Cbl associates with the SH2 domains of the adapter protein c-Crk and the Src family kinase Fyn. The specificity of these interactions is demonstrated by the fact that SH2 domains from other proteins, notably p85, are unable to bind c-Cbl in 3T3-L1 adipocytes after stimulation by insulin. *In vivo*, c-Cbl forms insulin-dependent complexes with c-Crk and Fyn. Although c-Crk showed a prominent insulin dependent association with c-Cbl, the significance of the c-Crk–c-Cbl interaction is difficult to ascertain because the cellular role of c-Cbl is unknown. We have recently demonstrated that c-Cbl is the major tyrosine-phosphorylated protein associated with c-Crk in haemopoietic cells [33]. This association is mediated exclusively through the SH2 domain of Crk, and is correlated with cellular transformation. The effect of insulin on c-Crk–c-Cbl binding is the first report of a hormone-induced c-Crk–c-Cbl association in non-transformed cells. Crk can constitutively associate with the nucleotide exchange proteins C3G and Sos through its SH3 domain [46,47]. Thus the formation of a c-Cbl–c-Crk complex after c-Cbl phosphorylation might target these exchange factors for the activation of $p21^{ras}$ or a member of the Ras family of GTPases such as Rab1 to recruit novel signalling pathways to the insulin receptor [48].

The significance of the c-Cbl–Fyn interaction is still unclear and remains to be elucidated. c-Cbl can interact with Fyn through both the SH2 and SH3 domains of the latter protein. These findings, together with the insulin-dependent association of c-Cbl with endogenous Fyn, suggest that c-Cbl might have a role in regulating the catalytic activity of Fyn in response to insulin in 3T3-L1 adipocytes. A similar mechanism of activation was proposed with Fyn and the p62-Sam68 protein [49,50].

To explore further the potential role of c-Cbl phosphorylation in insulin action, we observed that the tyrosine phosphorylation of c-Cbl was regulated during adipocyte differentiation. Insulin markedly induced the tyrosine phosphorylation of c-Cbl in the metabolic active 3T3-L1 adipocytes but not in 3T3-L1 fibroblasts, although insulin did stimulate the tyrosine phosphorylation of IRS-1 and Shc in 3T3-L1 fibroblasts. Interestingly, c-Cbl tyrosine phosphorylation was not observed after the stimulation of 3T3-L1 fibroblasts by IGF-1, although IRS-1 undergoes tyrosine phosphorylation under these conditions. Comparable amounts of c-Cbl are expressed in 3T3-L1 fibroblasts and adipocytes. Expression of the insulin receptor is lower in the fibroblasts than in the adipocytes, but is not decreased sufficiently to explain the difference in the phosphorylation of c-Cbl. Moreover, insulin failed to stimulate the tyrosine phosphorylation of c-Cbl in other cell lines expressing high levels of functional insulin receptors that have been used extensively to study insulin receptor signalling [4,14,37].

Shc and IRS-1 have recently been demonstrated to interact directly with the tyrosine-phosphorylated insulin receptor through specific N-terminal sequences of Shc or IRS-1 known as the PTB domain [51,52]. Although these substrates are generally not co-immunoprecipitated with the insulin receptor, their interaction has been extensively studied by two hybrid experiments with yeast [51,52], and with phosphorylated peptides modelled on the PTB-binding site in the insulin receptor [53]. Interestingly, the primary sequence of c-Cbl does not contain any motifs that might predict its interaction with the insulin receptor, such as an SH2 domain or the signature sequences commonly found in the PTB domain [53]. In this regard, we could not detect a direct association between c-Cbl and the insulin receptor or IRS-1, nor was c-Cbl detected in insulin receptor or IRS-1 immunoprecipitates (results not shown). Moreover, c-Cbl and the insulin receptor did not interact in two hybrid assays in yeast (results not shown). Thus c-Cbl seems not to interact directly with the insulin receptor. These findings suggest that the tyrosine phosphorylation of c-Cbl might require a specific accessory protein uniquely expressed in differentiated 3T3-L1 cells. Alternatively, it is possible that c-Cbl is not a direct substrate of the insulin receptor but is tyrosine phosphorylated by another tyrosine kinase present and activated in 3T3-L1 adipocytes.

The molecular events that lead to the specific tyrosine phosphorylation of c-Cbl in 3T3-L1 adipocytes are currently under investigation, as are the other cellular pathways that might be regulated by this phosphorylation.

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Received 6 December 1996/20 February 1997; accepted 25 February 1997