# Insulin stimulates the tyrosine dephosphorylation of docking protein p130$^{cas}$ (Crk-associated substrate), promoting the switch of the adaptor protein Crk from p130$^{cas}$ to newly phosphorylated insulin receptor substrate-1

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The docking protein p130$^{cas}$ (Crk-associated substrate) forms a stable complex with the adaptor protein CrkII in a tyrosine-phosphorylation-dependent manner. Insulin-induced tyrosine phosphorylation of insulin receptor substrates results in the redistribution of CrkII between p130$^{cas}$ and insulin receptor substrate-1. A decrease in the association between CrkII and p130$^{cas}$ in response to insulin stimulation was detected in CHO cells stably expressing insulin receptor or insulin receptor substrate-1, and in L6 rat myoblasts. Along with the decrease in the association of CrkII with p130$^{cas}$, the amount of tyrosine-phosphorylated insulin receptor substrate-1 co-precipitated with CrkII increased in all cell types studied. The insulin-induced decrease in the CrkII–p130$^{cas}$ association was further confirmed by Far Western Blot analysis with the Src homology 2 (SH2) domain of CrkII. Insulin regulates the association of CrkII with p130$^{cas}$ by tyrosine dephosphorylation of p130$^{cas}$ and co-ordinated tyrosine phosphorylation of insulin receptor substrate-1. Tyrosine-phosphorylated insulin receptor substrate-1 serves as a docking protein for multiple adaptor proteins and competes with p130$^{cas}$ for CrkII.

## INTRODUCTION

Adaptor proteins containing Src homology 2 (SH2) and Src homology 3 (SH3) domains link the insulin receptor to Ras signalling pathways [1,2]. Our recent observations have demonstrated that adaptor protein CrkII plays an important role in intracellular signalling mechanisms mediated by insulin [3]. Insulin treatment induces the formation of a complex between insulin receptor substrate-1 (IRS-1) and CrkII, mediated by the CrkII SH2 domain, in rat skeletal muscle L6 myoblasts [3]. Although several new classes of signalling molecules have been identified as Crk-binding proteins [4], the networks of interaction of Crk-binding proteins and their role in insulin signalling remain unknown at this time.

Considerable evidence indicates that the Crk-associated substrate (CAS or p130$^{cas}$), which contains an SH3 domain and numerous potential Crk-SH2 recognition sites, serves as a docking protein for the adaptor protein CrkII [5]. The association of CrkII with p130$^{cas}$ requires tyrosine phosphorylation of the docking protein. p130$^{cas}$ was originally identified as a major phosphotyrosine-containing protein in v-*crk*-transformed cells [6]. It is likely that Fyn kinase plays an essential role in the v-Crk-mediated phosphorylation of p130$^{cas}$ [7]. Stimulation of p130$^{cas}$ tyrosine phosphorylation by nerve growth factor, bombesin, lysophosphatidic acid, phorbol esters and platelet-derived growth factor (PDGF) was shown to lead to the formation of a p130$^{cas}$–CrkII complex [8,9]. In contrast, the dissociation of CrkII from pre-phosphorylated p130$^{cas}$ was reported in response to epidermal growth factor (EGF) in PC12 cells, possibly due to a higher affinity of the CrkII SH2 domain for newly phosphorylated p120$^{cbl}$ [10]. p130$^{cas}$ has been implicated in signalling pathways mediated by integrins. Integrin-mediated cell adhesion promotes the tyrosine phosphorylation of p130$^{cas}$ [11], and therefore p130$^{cas}$ can be regarded as a focal adhesion protein. p130$^{cas}$ is one of the proteins that interacts with focal adhesion kinase (FAK), a non-receptor protein-tyrosine kinase implicated in the control of cellular responses triggered by integrins [12]. FAK is not necessary for tyrosine phosphorylation of p130$^{cas}$, but may recruit Src-family kinases to phosphorylate p130$^{cas}$ [13]. Furthermore, the subcellular localization of the FAK–p130$^{cas}$ complex was influenced by Src [14]. Both the SH3 domain and the Src-binding domain of p130$^{cas}$ are required for its efficient localization to focal adhesions [15].

IRS proteins provide a common interface between insulin, cytokine receptors and various signalling proteins with SH2 domains [2]. Tyrosine-phosphorylated IRS-1 binds phosphatidylinositol 3-kinase, the phosphotyrosine phosphatase (PTPase) SHP2, the cytoplasmic tyrosine kinase Fyn, and the adaptor proteins Grb2, Nck and CrkII [1,3,16–20]. In the present study, we examined how insulin regulates the association of CrkII with two docking protein, p130$^{cas}$ and IRS-1. It seems that newly tyrosine-phosphorylated IRS-1 can displace pre-existing tyrosine phosphorylated p130$^{cas}$ from complexes with Crk.

## MATERIALS AND METHODS

### Materials

Recombinant human insulin and bovine pancreatic insulin were purchased from Sigma (St. Louis, MO, U.S.A.) and were used at concentrations between 0.85 and 1.7 $\mu$M, with similar results. Recombinant PDGF-BB and recombinant EGF were from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Tissue culture media and fetal calf serum were from Sigma and from Life Technologies (Gaithesburg, MD, U.S.A.). Protein A–Sepharose beads were from Pharmacia Biotech (Uppsala, Sweden). Protein A–horseradish peroxidase (HRP) conjugate and goat anti-(rabbit IgG)–HRP conjugate were from Bio-Rad Laboratories

Abbreviations used: Cas or p130$^{cas}$, Crk-associated substrate; IRS, insulin receptor substrate; SH2, Src homology 2; CHO cells, Chinese hamster ovary cells; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; HRP, horseradish peroxidase; FAK, focal adhesion kinase; PTPase, phosphotyrosine phosphatase; PTP-PEST, PTPase–PEST; GST, glutathione S-transferase; IGF-1, insulin-like growth factor-1.

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(Hercules, CA, U.S.A.), rabbit anti-(goat IgG)–HRP conjugate was from Zymed Laboratories (South San Francisco, CA, U.S.A.), and enhanced chemiluminescence detection reagents were from Amersham International (Amersham, Bucks., U.K.). Prestained protein molecular mass markers were from Bio-Rad and from New England Biolabs Inc. (Beverly, MA, U.S.A.). Acrylamide/Bis solution (29:1) was from Bio-Rad, and other electrophoresis materials and salts were from Fisher Scientific (Pittsburgh, PA, U.S.A.).

### Cell culture, cell lysis, immunoprecipitation and immunoblotting

L6 C6 is a clone of L6 rat myoblasts overexpressing mouse CrkII. The cloning of mouse CrkII, transfection of L6 cells and the establishment and characterization of the L6 C6 cell line were described previously [3]. Parental Chinese hamster ovary (CHO) cells and CHO cells expressing insulin receptor (CHO IR) or IRS-1 (CHO IRS-1) were described previously, and were a gift from M. White (Howard Hughes Medical Institute, Joslin Diabetes Center, Boston, MA, U.S.A.). Serum starvation, insulin stimulation, cell lysis, immunoprecipitation, Western blotting and Far Western blotting were performed exactly as described previously [3], with the exception that rabbit anti-(goat IgG)–HRP conjugate was used instead of protein A–HRP conjugate when primary antibodies were of goat origin.

### Antibodies

Rabbit polyclonal anti-IRS-1 and anti-IRS-2 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, U.S.A.) and Transduction laboratories (Lexington, KY, U.S.A.). Antibodies against CrkII were generated as described previously [3]. Rabbit polyclonal anti-CrkII antibodies purchased from Santa Cruz Biotechnology were used with similar results. Monoclonal anti-phosphotyrosine antibodies conjugated to HRP (Anti-P-Tyr) were purchased from Oncogene Research Products (Cambridge, MA, U.S.A.) and were used according to the manufacturer's protocols. Rabbit polyclonal anti-phosphotyrosine antibodies were used when specified. Goat polyclonal anti-Cbl antibodies (C-15) and agarose conjugate of rabbit polyclonal antibodies against Cbl were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-Cas antibodies raised against a peptide at either the N-terminus or the C-terminus of Cas were used for immunoprecipitation and immunoblotting. Both antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal anti-(rat p130$^{cas}$) antibodies from Upstate Biotechnology (Lake Placid, NY, U.S.A.) were used with similar results. Antibodies against paxillin were from Santa Cruz. Rabbit polyclonal antibodies against glutathione S-transferase (GST) (Upstate Biotechnology, Lake Placid, NY, U.S.A.) were used to detect GST fusion proteins in Far Western blotting.

## RESULTS

We have shown previously that CrkII binds to tyrosine-phosphorylated IRS-1 and plays an important role in insulin-dependent signalling [3]. We obtained further support for this notion in the present study using CHO cells and CHO cells overexpressing either insulin receptor (CHO IR) or IRS-1 (CHO IRS-1). Because CHO cells exhibit relatively high levels of endogenous CrkII (when compared with mouse 3T3 fibroblasts or L6 rat myoblasts), we have chosen this cell line to study insulin-mediated effects upon the association of CrkII with tyrosine-phosphorylated proteins. Tyrosine-phosphorylated IRS-1 was shown to co-immunoprecipitate with anti-Crk antibodies from insulin-stimulated CHO, CHO IR and CHO IRS-1 cells (Figure 1). As shown in Figure 1, another tyrosine-phosphorylated protein with an apparent molecular mass of 130 kDa was in complex with CrkII in unstimulated cells, and its association with CrkII was decreased after insulin stimulation (1.7 $\mu$M, 5 min). The decrease in CrkII association with this protein was dramatic. These results prompted us to investigate the nature of the co-precipitated 130 kDa protein and the mechanism responsible for the decrease in the amount of tyrosine-phosphorylated 130 kDa protein complexed with CrkII in insulin-treated cells.

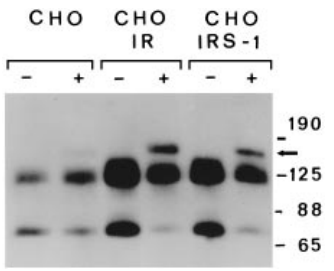


**Figure 1 Association of CrkII with tyrosine-phosphorylated proteins in CHO cells**

Lysates from insulin-stimulated (+) or unstimulated (—) cells were immunoprecipitated with anti-Crk antiserum. The resulting precipitates were separated by SDS/PAGE and analysed by Western blotting with anti-*P*-Tyr antibodies. Positions of molecular mass markers (in kDa) are shown on the right. The phosphorylated band corresponding to IRS-1 is indicated by the arrow, and can be seen clearly in stimulated cells overexpressing IR (CHO IR) or IRS-1 (CHO IRS-1) and faintly in the lane corresponding to stimulated parental cells (CHO).

To gain insights into the mechanism of association of Crk with the 130 kDa protein, we used the Far Western blotting method utilizing fusion proteins representing different domains of CrkII (Figure 2). As shown in Figure 2 (right-hand panels), both the fusion protein of complete CrkII and the fusion protein of its SH2 domain exhibited decreased binding to the 130 kDa protein in insulin-treated cells (1.7 $\mu$M, 5 min). This suggests that the interaction between CrkII and the 130 kDa protein was direct, was mediated by the CrkII SH2 domain and was not regulated via modification of the adaptor protein CrkII. It is likely that regulatory modification of the 130 kDa protein decreased the association of this protein with CrkII in response to insulin. We next used L6 rat myoblasts to determine the nature of the protein whose association with CrkII is negatively regulated by insulin.

L6 rat myoblasts have been well characterized in terms of insulin action [21,22]. We have shown previously that insulin treatment of L6 cells induces the formation of a stable complex of endogenous CrkII with IRS-1 mediated via the SH2 domain of Crk, and that the overexpression of CrkII enhanced the insulin-induced activation of extracellular-signal-regulated kinase (ERK) when compared with the parental cell line [3]. We have also found that insulin treatment caused a decreased association of CrkII with the phosphotyrosine-containing 130 kDa protein in L6 myoblasts. It was reported that, among the phosphoproteins migrating at 120–130 kDa in Rat1 fibroblasts, only FAK exhibits insulin-induced tyrosine dephosphorylation [23]. However, directly contrasting data indicating that FAK is tyrosine-phosphorylated during insulin-like growth factor-1 (IGF-1)-stimulated lamellipodial advance have been reported [24]. Since CrkII is recruited to the FAK–p130$^{cas}$ complex, the identity of the co-precipitated phosphorylated protein in L6 myoblasts had to be examined.

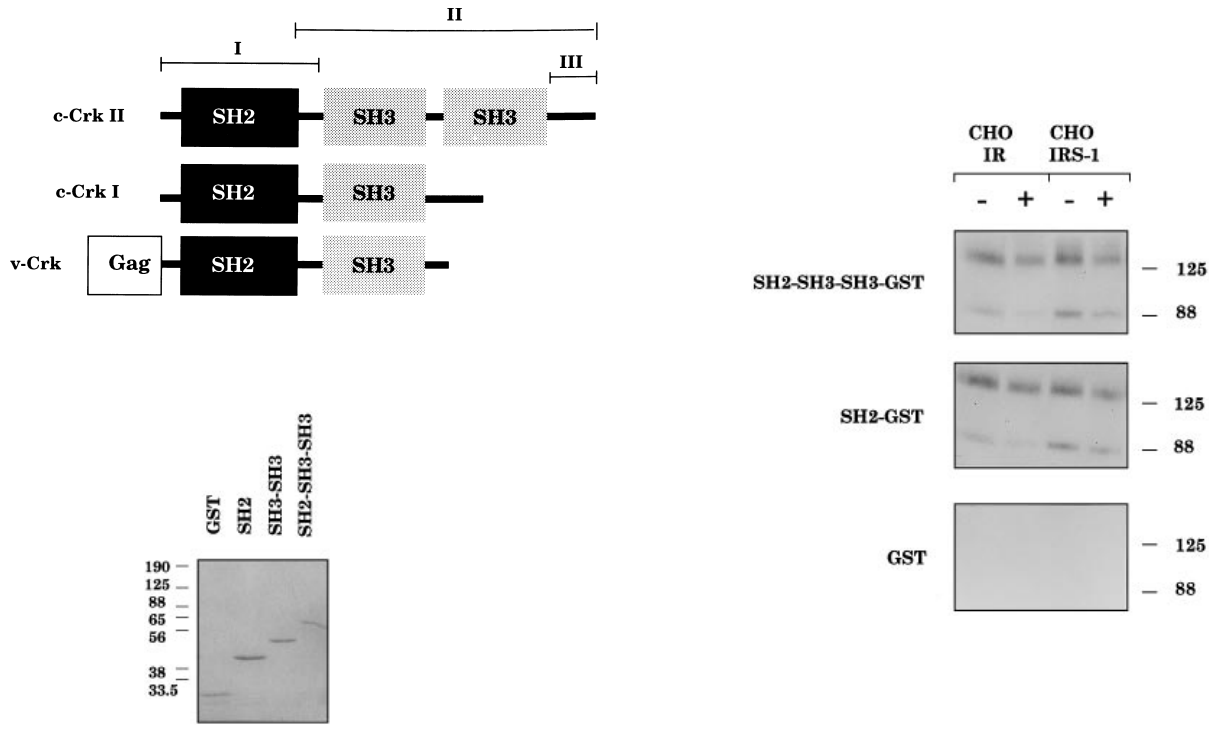


**Figure 2 Binding of the SH2 domain of Crk to tyrosine-phosphorylated proteins**

Top left: members of the Crk family of proteins and schematic representation of Crk fusion proteins used in this study. The modular binding domains are represented by black (SH2) and grey (SH3) boxes. The portions of mouse c-Crk (analogue of human c-CrkII) used for the generation of fusion proteins containing either SH2 domain (I) or two SH3 domains (II) are indicated. The position of a peptide used for rabbit immunization to produce anti-Crk antibodies is also indicated (III). Bottom left: fusion proteins used in this study. Fusion proteins were eluted from glutathione–Sepharose beads prior to SDS/PAGE analysis and Coomassie Blue staining. The GST–Crk fusion proteins are identified in relation to their modular binding domains. Positions of molecular mass markers (in kDa) are shown on the left. Right: Far Western blot analysis of insulin-stimulated (+) or unstimulated (−) CHO IR and CHO IRS-1 cells. Positions of molecular mass markers (in kDa) are shown on the right. The lysates separated by SDS/PAGE were probed with GST–Crk fusion proteins containing either complete Crk (SH2-SH3-SH3-GST) or the Crk SH2 domain (SH2-GST). As a control, the cell lysates were probed with GST. To highlight the differences in association of Crk fusion proteins with 130 kDa proteins, results obtained with a short time of film exposure are shown. With longer exposure times, binding of Crk fusion proteins to tyrosine-phosphorylated IRS-1 (170 kDa) was evident.

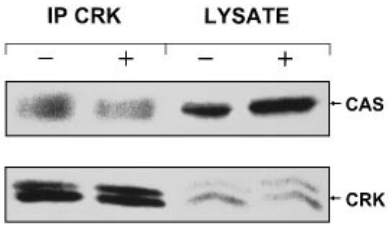


**Figure 3 Association of CrkII with p130$^{cas}$**

Lysates from insulin-stimulated (+) or unstimulated (−) L6 C6 cells were either separated by SDS/PAGE (Lysate) or subjected to immunoprecipitation with anti-Crk antiserum (IP Crk). The resulting precipitates were separated by SDS/PAGE and analysed by Western blotting with anti-Cas and anti-CrkII antibodies. The positions of p130$^{cas}$ and CrkII are indicated. The experiment presented is representative of three experiments.

Verification of the identity of the co-precipitated band was obtained by Western blot analysis with anti-Cas antibodies. Figure 3 shows that p130$^{cas}$ is associated with CrkII in quiescent L6 C6 cells and that insulin stimulation (0.85 $\mu$M, 5 min) results in a decrease in the amount of p130$^{cas}$ co-precipitated with anti-Crk antibodies. Similar results have been obtained in the parental L6 cell line. p130$^{cas}$ was highly tyrosine-phosphorylated in serum-restricted L6 C6 cells, whereas after the addition of insulin it was partially dephosphorylated (Figure 4). Equal amounts of p130$^{cas}$ were immunoprecipitated from quiescent and insulin-treated cells. To prove that p130$^{cas}$ is a major tyrosine-phosphorylated protein associated with CrkII in the basal state, we performed CrkII co-precipitation experiments with p130$^{cas}$-depleted L6 lysates. For immunodepletion experiments, equal aliquots of L6 lysates either were subjected immediately to immunoprecipitation with anti-CrkII antibodies or were immunodepleted using three serial immunoprecipitations with a preformed complex of anti-Cas and Protein A–Sepharose prior to immunoprecipitation with anti-CrkII antibodies (Figure 5). The resulting disappearance of p130$^{cas}$ from L6 lysates was verified by Western blot analysis of treated aliquots of L6 lysates as well as of anti-Cas immunoprecipitates. A third subsequent immunoprecipitation with anti-Cas–Protein A–Sepharose failed to precipitate any p130$^{cas}$. In parallel, p130$^{cas}$ was not detected in L6 lysates, and subsequent immunoprecipitation with anti-CrkII antibodies did not co-precipitate any tyrosine-phosphorylated protein of molecular mass about 130 kDa (Figure 5). We conclude that the 130 kDa protein that is associated with CrkII and is regulated by insulin is p130$^{cas}$. The fact that p130$^{cas}$ was phosphorylated on tyrosine in the absence of insulin can be explained by adhesion-induced tyrosine phosphorylation [11,25]. We obtained additional evidence that p130$^{cas}$ was highly phosphorylated in serum-restricted L6 cells by immunoprecipitating Cas from

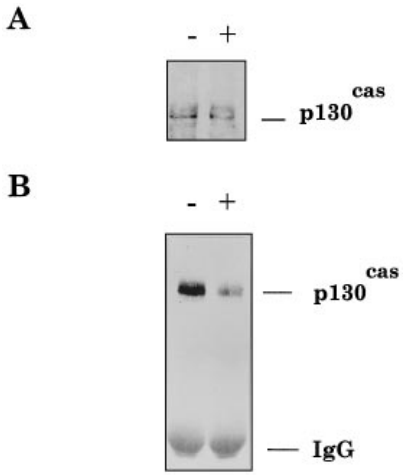


**Figure 4 Insulin stimulates the tyrosine dephosphorylation of p130$^{cas}$**

Lysates from insulin-stimulated (+) or unstimulated (−) L6 cells were subjected to immunoprecipitation with anti-Cas antibodies. The resulting precipitates were separated by SDS/PAGE and analysed by western blotting either with anti-Cas antibodies (**A**) or with anti-*P*-Tyr antibodies (**B**). The positions of p130$^{cas}$ and IgG are indicated.

[$^{32}$P]$P_i$-labelled cells (results not shown). p130$^{cas}$ has been found to be tyrosine-phosphorylated in some continuously adherent cells [26,27].

A switch of CrkII association from p130$^{cas}$ to p120$^{cbl}$, the product of the c-*cbl* proto-oncogene, was shown in response to EGF in PC12 cells [10]. To investigate whether p120$^{cbl}$ can play a similar role in insulin-stimulated L6 rat myoblasts, lysates from insulin-stimulated L6 cells were immunoprecipitated with antibodies against c-Cbl and the level of c-Cbl tyrosine phosphorylation was determined. Exposure of the cells to insulin (0.85 $\mu$M, 5 min) did not result in any increase in the tyrosine phosphorylation of p120$^{cbl}$ in L6 cells (Figure 6). Serum or PDGF stimulation (used as a positive control in this study) of the same cells resulted in a dramatic increase in the phosphorylation of p120$^{cbl}$. The absence of detectable insulin-induced tyrosine phosphorylation of p120$^{cbl}$ leaves no opportunity for c-Cbl to serve as an alternative binding partner for CrkII in insulin-stimulated L6 cells. After insulin stimulation, the SH2 domain of

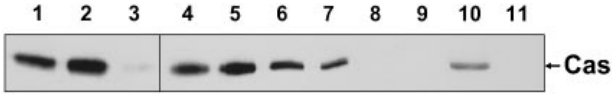


**Figure 5 Immunodepletion of L6 cell lysates using anti-Cas antibodies**

Lysates from insulin-stimulated (5 min) (lane 1) and unstimulated (lanes 2 and 3) L6 cells either were subjected to immunoprecipitation with anti-CrkII antiserum (lanes 1 and 2) or were first immunodepleted with anti-Cas antibodies prior to immunoprecipitation with anti-Crk antiserum (lane 3). Lanes 1–3 represent Western blot analysis using polyclonal anti-*P*-Tyr antibodies. Immunodepletion was performed by three serial immunoprecipitations with a preformed complex between anti-Cas and Protein A–Sepharose. The resulting precipitates from the first (lanes 4 and 5), second (lanes 6 and 7) and third (lanes 8 and 9) immunoprecipitations of the same lysates were analysed by Western blotting with anti-Cas antibodies. Because of successful immunodepletion, the third immunoprecipitate does not contain Cas protein (lanes 8 and 9). Western blot analysis using anti-Cas antibodies of L6 cell lysates before (lane 10) and after (lane 11) immunodepletion is also presented. Lanes 4, 6 and 8 represent the immunodepletion of unstimulated L6 lysate, whereas lanes 5, 7 and 9 correspond to insulin-stimulated L6 cells.

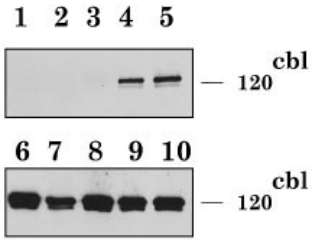


**Figure 6 p120$^{cbl}$ is tyrosine-phosphorylated in response to serum and PDGF, but not insulin, in L6 C6 cells**

Lysates from unstimulated (lanes 1 and 6), insulin-stimulated (lanes 2 and 7), EGF-stimulated (lanes 3 and 8), serum-stimulated (lanes 4 and 9) and PDGF-stimulated (lanes 5 and 10) cells were subjected to immunoprecipitation with an agarose conjugate of rabbit polyclonal antibodies against Cbl. The resulting precipitates were separated by SDS/PAGE (10% gel) and analysed by Western blotting either with anti-*P*-Tyr antibodies (lanes 1–5) or with goat polyclonal antibodies against Cbl (lanes 6–10). The position of p120$^{cbl}$ is indicated.

CrkII most likely interacts with newly tyrosine-phosphorylated IRS proteins.

In addition to IRS-1, insulin is known to stimulate the tyrosine phosphorylation of IRS-2, another member of the family of docking proteins with multiple phosphorylation sites serving as targets for SH2 domains [2]. Since IRS-1 and IRS-2 share significant structural similarity, the selectivity of the CrkII–IRS-1 interaction was tested using L6 C6 cells. Overexpressed CrkII was immunoprecipitated and the electrophoretic mobility of the co-precipitated tyrosine-phosphorylated protein was compared with the positions of phosphorylated IRS-1 and IRS-2 (Figure 7). The prolonged duration of electrophoretic separation highlighted the differences in electrophoretic mobilities of immunoprecipitated IRS-1 and IRS-2 in rat myoblasts, and enabled us to confirm the CrkII–IRS-1 interaction. Figure 7 demonstrates that tyrosine-phosphorylated IRS-1 (and not IRS-2) is co-precipitated with anti-Crk antibodies. To obtain more convincing evidence that CrkII binds selectively to IRS-1 and not IRS-2 following insulin stimulation (5 min, 0.85 $\mu$M), we performed a Far Western blot of IRS-2 precipitates with the Crk SH2 domain fusion protein (Figure 8). Anti-Cas precipitate from unstimulated cells was used as a positive control. No binding of the Crk SH2 domain to immunoprecipitated IRS-2 was detected under our experimental conditions.

We attempted to identify the nature of a 75 kDa tyrosine-phosphorylated protein that was sometimes detected in CrkII immunoprecipitates (Figure 1 and [3]). Association of v-Crk through its SH2 domain with paxillin have been reported [28]. It is intriguing that insulin stimulation of fibroblasts and CHO cells induces the rapid tyrosine dephosphorylation of paxillin [29,30]. Therefore we immunoprecipitated paxillin from L6 cells and performed Far Western blot analysis with the fusion protein of the Crk SH2 domain (Figure 8). No binding of the Crk SH2 domain to immunoprecipitated paxillin was detected under our experimental conditions.

## DISCUSSION

The exchange of one docking protein for another in response to a physiological stimulus was reported previously for CrkII and proteins p120$^{cbl}$ and p130$^{cas}$ after EGF stimulation of PC12 cells [10].

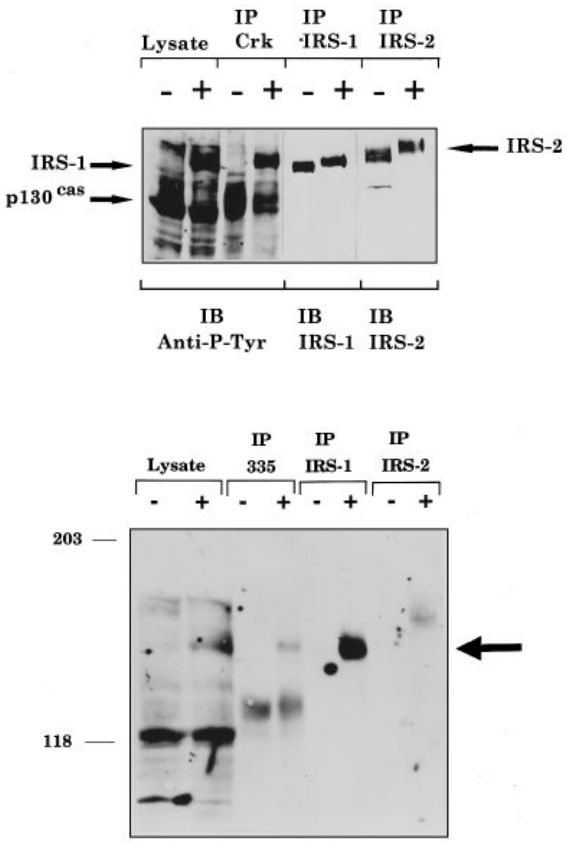


**Figure 7 Association of CrkII protein with IRS-1 in L6 C6 cells**

Upper panel: lysates of insulin-stimulated (+) or unstimulated (−) cells were either separated by SDS/PAGE (Lysate) or subjected to immunoprecipitation with anti-Crk (IP Crk), anti-IRS-1 (IP IRS-1) or anti-IRS-2 (IP IRS-2) antiserum before separation. The resulting precipitates and lysates were analysed by Western blotting with anti-*P*-Tyr antibodies (IB Anti-*P*-Tyr), anti-IRS-1 (IB IRS-1) or IRS-2 (IB IRS-2) antibodies. The positions of bands corresponding to IRS-1, IRS-2 and p130$^{cas}$ are indicated by arrows. Lower panel: in a similar experiment the prolonged duration of SDS/PAGE separation highlighted the differences in electrophoretic mobilities of immunoprecipitated IRS-1 and IRS-2. The lane termed IP 335 corresponds to immunoprecipitation with polyclonal anti-Crk antibodies [3]. Lysates and immunoprecipitates were analysed by Western blotting with anti-*P*-Tyr antibodies. The position of a band corresponding to IRS-1 is indicated by an arrow. Positions of molecular mass markers (in kDa) are shown on the left.

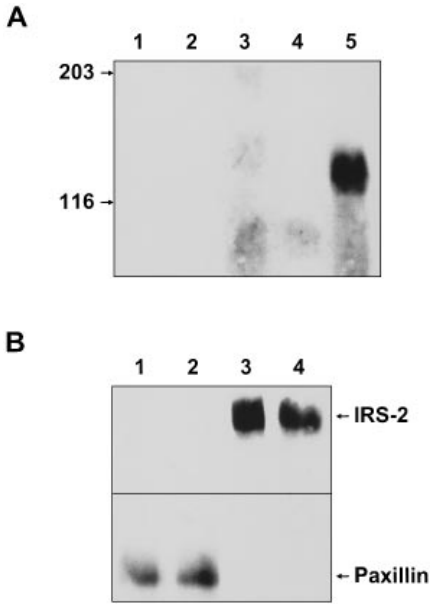


**Figure 8 Far Western blot analysis of insulin-stimulated and unstimulated L6 cells**

(**A**) Paxillin (lanes 1 and 2), IRS-2 (lanes 3 and 4) and Cas (lane 5) were immunoprecipitated from insulin-stimulated (lanes 2 and 4) and unstimulated (lanes 1, 3 and 5) L6 cells and, after separation by SDS/PAGE, were probed with GST–Crk fusion protein containing the SH2 domain. Positions of molecular mass markers (in kDa) are shown on the left. (**B**) To verify the positions of IRS-2 and paxillin, the nitrocellulose was stripped and blotted with anti-IRS-2 (upper panel) and anti-paxillin (lower panel) antibodies. The positions of IRS-2 and paxillin are indicated.

The present paper is the first report of an insulin-induced redistribution of an adaptor protein between two docking proteins. Although insulin induced tyrosine phosphorylation of c-Cbl in 3T3-L1 adipocytes, neither IGF-1 nor insulin stimulated the tyrosine phosphorylation of c-Cbl in 3T3-L1 fibroblasts or hepatocytes [26]. In the present study, we demonstrate that p120$^{cbl}$ is not tyrosine-phosphorylated in response to insulin (5 min exposure) in L6 rat myoblasts, and that newly tyrosine-phosphorylated IRS-1 competes with p130$^{cas}$ for the CrkII SH2 domain. It is interesting to note that both IRS-1 and IRS-2 were initially described as a specific binding partner for CrkII in NIH 3T3 and 293 cells stably transfected with an expression vector containing CrkII cDNA [19]. One possible explanation is that the level of IRS-2 tyrosine phosphorylation in L6 cells treated with insulin is lower when compared with IRS-1 phosphorylation, or with IRS-2 phosphorylation in NIH 3T3 and 293 cells treated with IGF-1. It was reported that IRS-2 was rapidly (3–10 min) dephosphorylated after the addition of insulin in L6 cells, whereas IRS-1 phosphorylation was maintained for at least 60 min [31]. However, the fact that the association of both IRS proteins with CrkII was shown to decrease in NIH 3T3 and 293 cells on IGF-1 treatment [19] could indicate that another type of interaction, different from the direct interaction mediated by the CrkII SH2 domain, also takes place. An alternative explanation of the reported decrease of the association of CrkII with IRS-1 and IRS-2 is ligand-induced modification of CrkII [19]. Our data support the view that IRS-1 and IRS-2 play unique roles in mediating the signals from the insulin receptor to downstream molecules [31]. Disruption of IRS-1 in mice causes insulin resistance, but does not impair the compensatory insulin production [32]. In contrast, disruption of IRS-2 causes type 2 diabetes in mice [33], suggesting that dysfunction of IRS-2, but not IRS-1, contributes to the pathophysiology of type 2 diabetes. Nevertheless, it appears that amino acid polymorphisms in IRS-2 are not associated with random type 2 diabetes in humans [34]. Thus it is still not clear whether signalling through IRS-1 or signalling through IRS-2 is of primary significance for the pathophysiology of type 2 diabetes in humans.

Rapid dephosphorylation of p130$^{cas}$ could result from the insulin-induced activation of PTPases. A small increase in total PTPase activity was induced by insulin in L6 rat fibroblasts [35]. Furthermore, immunoprecipitated PTPase 1B showed increased phosphatase activity within 30 min after the insulin [35]. PTPase 1B is one of the phosphatases that has a major influence on insulin action [36]. It is of note that direct binding of the proline-rich region of PTPase 1B to the SH3 domain of p130$^{cas}$ has been demonstrated [37]. Recently p130$^{cas}$ was identified as a preferred substrate *in vitro* and *in vivo* for another PTPase, PTP-PEST (PTPase–PEST) [38]. The interaction between p130$^{cas}$ and PTP-PEST involves two distinct substrate recognition mechanisms:

specificity is dictated by the catalytic domain of PTP-PEST, whereas the efficiency of association is determined by binding of the p130$^{cas}$ SH3 domain to the proline-rich sequence within the non-catalytic segment of PTP-PEST [39]. Thus at least two PTPases have been shown to be associated with p130$^{cas}$. It was shown previously that insulin stimulates the tyrosine dephosphorylation of FAK and paxillin [23,29]. Insulin-induced tyrosine dephosphorylation of FAK and paxillin in mouse fibroblasts required active PTPase 1D [29]. It is intriguing that all phosphoproteins that have been shown to undergo tyrosine dephosphorylation in response to insulin (FAK, paxillin and p130$^{cas}$) belong to the family of focal adhesion proteins, whereas IRS proteins tyrosine phosphorylated in response to insulin are involved in transferring growth-related and metabolic signals.

Because skeletal muscle is one of the major sites of tissue insulin resistance in obesity and diabetes with respect to glucose disposal [40], our results obtained with skeletal muscle L6 myoblasts are of importance for understanding insulin signalling in obesity and in the diabetic state. Using a phosphorylated peptide derived from the kinase domain of the insulin receptor, total PTPase activity was evaluated in skeletal muscle from non-diabetic obese subjects [41]. Both the total PTPase activity and the level of PTPase 1B were reported to be decreased in these obese subjects [41]. However, when using the intact insulin receptor protein as substrate and analysing a different (with regard to overweight) population of obese subjects, PTPase activity in skeletal muscle was shown to be increased in insulin-resistant obese non-diabetic subjects when compared with lean controls [42]. In contrast, PTPase activity in skeletal muscle from subjects with non-insulin-dependent diabetes was significantly decreased when compared with the level in controls in both studies [41,42]. Further studies will be necessary to determine the phosphorylation status of p130$^{cas}$ in skeletal muscle tissue from diabetic and non-diabetic obese subjects.

Our data suggest that insulin could regulate intracellular signalling pathways via the recruitment of adaptor protein CrkII to alternative docking proteins IRS-1 or p130$^{cas}$ by co-ordinating the processes of tyrosine phosphorylation and dephosphorylation. The docking protein p130$^{cas}$ is implicated in the assembly of focal adhesions, whereas IRS-1 serves as a common interface between several tyrosine kinase receptors and various signalling proteins involved in growth-related signalling. It remains to be determined whether this switch represents the blunting of signalling pathways mediated by integrins in favour of tyrosine-kinase-receptor-mediated signalling.

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