Interaction *in vitro* of the product of the c-Crk-II proto-oncogene with the insulin-like growth factor I receptor

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The Crk proto-oncogene product is an SH2 and SH3 domain-containing adaptor protein. We have previously demonstrated that Crk-II becomes rapidly tyrosine-phosphorylated in response to stimulation with insulin-like growth factor I (IGF-I) and might be involved in the IGF-I receptor signalling pathway. To determine whether this involvement includes the direct interaction of Crk-II with the cytoplasmic region of the receptor, studies were performed *in vitro* with glutathione S-transferase (GST) fusion proteins containing various domains of Crk-II. The kinase assay *in vitro* showed that activated IGF-I receptors efficiently phosphorylated the GST–Crk-II fusion protein. This phosphorylation was dependent on the presence of the SH2 domain and Tyr-221 located in the spacer region between the two SH3 domains. Mutation of Tyr-221 not only prevented phosphorylation of GST–Crk *in vitro*, but also significantly increased the ability of GST–Crk proteins to co-precipitate activated IGF-I receptors from total cell lysates. Additional binding experiments *in vitro* showed that Crk-II might interact with the phosphorylated IGF-I receptor through its SH2 domain. To elucidate which

region of the IGF-I receptor interacts with Crk-II, a peptide association assay was used *in vitro*. Different domains of the IGF-I receptor were expressed as (His)₆-tagged fusion peptides, phosphorylated with activated wheat germ agglutinin-purified IGF-I receptors and tested for association with GST–Crk-II fusion proteins. Using wild-type as well as mutated peptides, we showed that the SH2 domain of Crk-II preferentially binds the peptide encoding the juxtamembrane region of the IGF-I receptor. Phosphorylation of Tyr-950 and Tyr-943 of the receptor is important for this interaction. These findings allow us to propose a model of direct interaction of Crk-II and the IGF-I receptor *in vivo*. On activation of the IGF-I receptor, Crk-II binds to phosphorylated tyrosine residues, especially in the juxtamembrane region. As a result of this binding, the IGF-I receptor kinase phosphorylates Tyr-221 of Crk-II, resulting in a change in intramolecular folding and binding of the SH2 domain to the phosphorylated Tyr-221, which causes rapid disassociation of the Crk-II–IGF-I receptor complex.

INTRODUCTION

The insulin-like growth factor I (IGF-I) receptor is a member of the tyrosine kinase receptor family [1–3] and is composed of two α and two β subunits. The α subunits are entirely extracellular and bind IGF-I and IGF-II with high affinity. The two transmembrane β subunits contain tyrosine kinase domains that are activated after ligand binding. Disulphide bonds link the α and β subunits; two $\alpha\beta$ hemireceptors are linked by disulphide bonds making an $\alpha_2\beta_2$ dimer that forms the functional receptor. The cytoplasmic portion of the β subunit of the receptor can be divided into three distinct domains: the juxtamembrane (JCT), tyrosine kinase and C-terminal (CT) domains. Ligand-stimulated receptor autophosphorylation is essential for full biological activity of the IGF-I receptor [4–11]. Initially, phosphorylation occurs on a cluster of conserved tyrosine residues (residues 1131, 1135 and 1136) in the tyrosine kinase domain [3]. Additional tyrosines that seem to be involved in IGF-I receptor signalling include Tyr-950 in the JCT domain [12] and Tyr-1250, Tyr-1251 and Tyr-1316 in the CT domain [13]. After activation of receptor tyrosine kinase, a number of substrates and signalling pathways are activated. IRS-1 and IRS-2 are rapidly phosphorylated on tyrosine residues [14–16], as are the cytosolic proteins p46Shc and p52Shc [17,18]. IRS-1, IRS-2 and Shc link the activated receptor to downstream signalling pathways. Tyrosine phos-

phorylation sites on IRS-1 and IRS-2 are binding sites for several SH2 domain-containing proteins including p85 (the regulatory subunit of PtdIns 3'-kinase) [19], Syp (tyrosine-specific phosphatase) [20] and Grb-2 (a small adaptor protein) [21]. Although both IRS-1 and Shc interact with the JCT domain via the NPEY motif surrounding residue Tyr-950, IRS-1 might also bind the major autophosphorylation site (Tyr-1131, Tyr-1135 and Tyr-1136) in the tyrosine kinase domain [22]. Activated Shc proteins apparently bind only Grb-2 [23]. Grb-2, whether bound to IRS-1 or Shc, binds to mSOS via its SH3 domain and activates the Ras/Raf/mitogen-activated protein (MAP) kinase pathway (reviewed in [14,16,24]).

Recent studies have revealed that in addition to IRS-1, IRS-2 and Shc, substrates such as Syp, p85 and GAP interact directly with the IGF-I receptor *in vitro* through their SH2 domains [25]. GAP binds to Tyr-950 in the JCT domain, whereas Syp and p85 interact with Tyr-1316 in the CT domain [25].

The Crk family consists of proteins that include Crk-I, Crk-II, v-Crk and CrkL, containing SH2 and SH3 domains with very short intervening sequences [26]. Despite the lack of a kinase domain these proteins are heavily phosphorylated *in vivo* and in intact cells on tyrosine residues [27,28] and apparently have an important role in growth factor-stimulated signal transduction [28–30]. The widely expressed Crk-II protein contains an N-terminal SH2 domain followed by two SH3 domains [26,31].

Abbreviations used: CT, C-terminal; EGF, epidermal growth factor; GST, glutathione S-transferase; IGF, insulin-like growth factor; JCT, juxtamembrane; MAP, mitogen-activated protein; NGF, nerve growth factor; PLC, phospholipase C; WGA, wheat germ agglutinin.

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Crk-I is an alternatively spliced version of Crk-II that lacks the C-terminal SH3 domain [26,31] and thus closely resembles the viral oncogenic form of Crk, v-Crk [32]. CrkL is a recently identified member of the family with one SH2 and two SH3 domains [33]. Both the SH2 and SH3 domains are essential for signal transduction [34].

We have demonstrated that the product of the c-Crk-II proto-oncogene is phosphorylated on tyrosine residues by the activated IGF-I receptor [28]. Crk-II directly binds the nucleotide exchange proteins C3G and mSOS, thereby activating the Ras/Raf/MAP kinase pathway [35]. Indeed, overexpression of Crk-II enhances the mitogenic response of NIH-3T3 cells to IGF-I [36]. These observations support a role for Crk-II in IGF-I receptor signalling [36].

To elucidate the molecular mechanisms involved in IGF-I receptor signalling through Crk-II, we used kinase assays and binding experiments *in vitro* to investigate the direct interaction of the IGF-I receptor and Crk-II. On the basis of the results presented here we conclude that this interaction might be direct and transient, and that the tyrosine phosphorylation of Crk-II might result in a conformational change of the molecule that causes this rapid disassociation of Crk-II from the IGF-I receptor.

EXPERIMENTAL

Cell culture and IGF-I stimulation

NIH-3T3 mouse fibroblasts overexpressing IGF-I receptors (NWTb3; approx. 5×10^5 receptors per cell) [4] were cultured in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Before stimulation with IGF-I, subconfluent cultures of cells in 100 mm dishes were serum-starved for 18–20 h in Dulbecco's modified Eagle's medium supplemented with 0.1% insulin-free BSA (Intergen, Purchase, NY, U.S.A.) and 20 mM Hepes, pH 7.5. The cells were then incubated either without or with 20–100 nM IGF-I (Genentech, Palo Alto, CA, U.S.A.) for various times, as indicated in the figure legends.

Purification of solubilized membranes with wheat germ agglutinin (WGA) and autophosphorylation *in vitro*

Confluent NIH-3T3 cells overexpressing the IGF-I receptor (NWTb3) were serum-starved for 20 h, washed with ice-cold PBS and harvested in lysis buffer containing 50 mM Hepes, pH 7.6, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF and 100 units/ml bacitracin. The solubilized receptors were partly purified over a WGA-agarose column as described previously [37]. Typically, the second, third and fourth 1 ml fractions eluted from the column were combined, concentrated with a Centricon-100 filter unit (Amicon, Beverly, MA, U.S.A.) to approximate concentrations of 100 µg/ml total protein and stored at -70°C . For autophosphorylation *in vitro*, WGA-purified receptors were thawed and the elution buffer was replaced with kinase buffer [50 mM Hepes (pH 7.5)/100 mM NaCl/5 mM MgCl_2 /5 mM MnCl_2 /0.1% (v/v) Tween-20/0.1% BSA/0.5 mM PMSF]. WGA-purified IGF-I receptors were stimulated for 1 h at 20°C in the presence of 100 nM IGF-I. Autophosphorylation was initiated by the addition of 200 µM ATP. The reaction continued for 15 min at 24°C and was stopped by the addition of EDTA to a final concentration of 20 mM. To prevent the autophosphorylation of WGA-purified IGF-I receptors, EDTA (final concentration 20 mM) was added to a reaction before the addition of ATP.

Expression and purification of glutathione S-transferase (GST) fusion proteins

GST–Crk expression constructs encoding the full-length SH3–SH3 domains, and the N-terminal SH3 and C-terminal SH3 domains of mouse Crk-II, were gifts from Dr. Beatrice Knudsen (Rockefeller University, New York, NY, U.S.A.). GST–Crk expression constructs containing the SH2 (residues 9–119), SH2–SH3 A (residues 9–208) and SH2–SH3 (residues 9–236) domains of Crk-II were obtained by subcloning the corresponding PCR fragment amplified with a plasmid encoding full-length mouse Crk-II as a template into a pGEX-5X-3 GST–gene fusion vector (Pharmacia Biotechnology, Piscataway, NJ, U.S.A.). The common upstream primer for the three PCR was 5'-GCG GGATCCCCGAGCGAGTAGCTGGTACT-3'. Corresponding 3' primers were (1) to amplify the SH2 domain, 5'-GGGG-GCGGCCGCTGGAAACTGGTTCTATCAACGT-3', (2) to amplify the region encoding 9–236 amino acids of Crk-II, 5'-CCCCGGATCCCATTCTGGAGGTTAGG-3' and (3) to generate the SH2–SH3A construct, 5'-CCCCGGATCCCCTACTG-AGGCGGAGGCAGGT-3'. Underlined sequences indicate *Bam*HI and *Not*I restriction enzyme sites. The expression construct GST–SH2 RV encoding a mutated form of the SH2 domain (replacement of Arg-38 by Val) and the construct GST–Y221F encoding a mutated form of full-length mouse Crk-II (replacement of Tyr-221 by Phe) were created by specific mutagenesis and recombination techniques similar to that described below. All constructs were sequenced to ensure in-frame ligations and the presence of the engineered mutations, and the resulting expressed proteins were tested by immunoblotting with anti-Crk II antibodies (Transduction Laboratories, Lexington, KY, U.S.A., and Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). This anti-Crk-II antibody recognized all constructs except the GST–CrkSH2 and the mutant GST–SH2 RV. The expression constructs encoding both SH2 domains of the p85 subunit of PtdIns 3'-kinase and the N-terminal and C-terminal SH2 domains of phospholipase C- γ (PLC γ) were a gift from Dr. Alan Saltiel (Parke Davis, Ann Arbor, MI, U.S.A.). Plasmid DNA species were transfected into *Escherichia coli* DH5 α (Gibco BRL, Gaithersburg, MD, U.S.A.). Bacterial colonies were grown at 37°C in 500 ml of Luria–Bertani broth to a D_{595} of 0.8–1.0, induced with 0.1 mM isopropyl β -D-thiogalactoside for 2–3 h and collected by centrifugation. Bacteria were lysed in 20 ml of lysis buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1% (v/v) Triton X-100 and 100 mM EDTA and sonicated briefly; the cell debris was removed by centrifugation at 16000 rev./min for 30 min. Supernatants were incubated with 1.5 ml of glutathione–Sepharose 4B beads (Pharmacia Biotechnology) for 4–5 h. Beads were washed three times with lysis buffer and twice in PBS. GST fusion proteins were eluted from the beads with neutralized 50 mM reduced glutathione in PBS with 5% (v/v) glycerol for 2 h. After dialysis overnight against the same buffer, eluates were adjusted to 1–2 µg/µl protein. The purity, integrity and concentrations of bacterially expressed GST–Crk fusion proteins were assessed by SDS/PAGE and Coomassie Blue staining. All proteins appeared as a single band of the expected molecular mass.

Kinase assay *in vitro*

Serum-starved NIH-3T3 cells overexpressing the IGF-I receptor were stimulated with 50 nM IGF-I for 3 min and lysed in RIPA buffer [20 mM Tris/HCl (pH 7.5)/100 mM NaCl/5% (v/v) glycerol/2.5 mM EDTA/1% (v/v) Triton X-100/0.5% deoxycholate/0.1% SDS/1 mM sodium orthovanadate/0.3 mM sodium molybdate/10 mM NaF, containing protease inhibitors

(2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin and 1 mM PMSF)]; IGF-I receptors were immunoprecipitated overnight from 100 μg of total protein with 1 μg of anti-IGF-I receptor β subunit) polyclonal antibody (antibody C-20; Santa Cruz Biotechnology) at 4 °C. A 50 μl sample of a 50% (w/v) suspension of Protein A-Sepharose beads was added and the incubation was continued for an additional 4 h. The beads containing the IGF-I receptor were washed twice with 1 ml of RIPA buffer and twice with 1 ml of kinase buffer without ATP. The beads were resuspended in 100 μl of kinase buffer containing 1 μg of GST fusion protein and 50 μM ATP. The reaction mixtures were incubated at room temperature for 15 min and centrifuged briefly to precipitate the beads. Then 30 μl aliquots were removed and mixed with 8 μl of 5 $\times$ loading buffer. The GST fusion proteins were separated by SDS/PAGE [9% (w/v) gel], transferred to nitrocellulose membranes and immunoblotted with an anti-phosphotyrosine antibody (4G10, diluted 1:2000) (Upstate Biotechnology). If necessary the membrane was stripped and reblotted with an anti-GST antibody diluted 1:2000 (Upstate Biotechnology), as indicated in the figure legends.

Expression and purification of peptides corresponding to the JCT and CT domains of the IGF-I receptor

Various plasmids encoding native and various mutated forms of the IGF-I receptor, created in our laboratory, were used as templates to amplify the JCT and CT domains of the receptor by PCR. Additional mutations were created by using specific mutagenesis and recombination with the PCR technique [38], with modifications. Primary PCR products were purified with a QIA quick spin PCR purification kit (Qiagen, Chatsworth, CA, U.S.A.), mixed in equimolar concentrations (3–5 μg of the longest PCR product) and subjected to ten thermal cycles. Outside primers (0.1 μg of each) were then added and PCR was continued for ten more cycles. Upstream primers, 5'-CGCGGATCCGT-TCTGAGATCATCAGC-3' and downstream primers, 5'-GGGGAATTCGAGGTCGAAGACTGGGGCAG-3', were used to amplify a sequence encoding residues 1229–1337 of the CT domain of the IGF-I receptor, whereas primers 5'-CGCGGATCCGAGAAAGAGAAATAACAGC-3' and 5'-GGGGAATTCCTGCCCAAGTTCCCGGCT-3' were used to amplify the sequence encoding residues 930–977 of the JCT region. The underlined sequences indicate *Bam*HI and *Eco*RI restriction sites. The PCR products were digested with *Bam*HI and *Eco*RI, purified and ligated into the pTrcHis-B bacterial expression vector (Invitrogen, San Diego, CA, U.S.A.), which contains a (His)₆-Tag upstream of its multiple cloning site. The ligation mixtures were then transfected into *E. coli* DH5 α . All expression constructs were sequenced to ensure in-frame ligation of the inserts and the presence of the mutations. The peptides were expressed and purified under denaturing conditions with the Xpress Purification System in accordance with the manufacturer's instructions (Invitrogen). Purity, integrity and concentration of the peptides were checked by SDS/PAGE and Coomassie Blue staining. The purity of all peptides was greater than 90% with one dominant band of the expected molecular mass, as detected by immunoblotting with an anti-T7-Tag antibody (diluted 1:5000) (Novagen, Madison, WI, U.S.A.). All fusion peptides consisted of (His)₆- and T7-Tag epitopes at the N-terminus or JCT or CT regions of the IGF-I receptor, and 18 vector-encoded residues at the C-terminus. Specific mutations in receptor coding sequences are discussed in the Results section. The identities of peptides containing the CT domain were confirmed by immunoblotting with an antibody to the β subunit

of the IGF-I receptor [Ab 1-2; a gift from Professor Ken Siddle (Cambridge, U.K.)].

Precipitations of IGF-I receptors and WGA-purified IGF-I receptors with GST fusion proteins

Total cell lysates (400–600 μg of total protein) in 400 μl of RIPA buffer were mixed with 400 μl of ice-cold binding buffer [50 mM Tris/HCl (pH 7.5)/100 mM NaCl/1 mM EDTA/0.1% (v/v) Tween-20/1% (w/v) BSA/10 μM sodium orthovanadate/10 μM sodium molybdate/0.5 mM PMSF]. GST protein (2 μg) or equimolar amounts of GST fusion proteins (4–6 μg) and 30 μl of glutathione-Sepharose 4B beads [50% (w/v) suspension in binding buffer] were added and incubated overnight at 4 °C. Samples were then washed three times with 1 ml of ice-cold washing buffer [10 mM Tris/HCl (pH 7.5)/100 mM NaCl/1 mM sodium orthovanadate/1 mM EDTA/1 mM EGTA/1% (v/v) Triton X-100/0.5% (v/v) Nonidet P40/0.2 mM PMSF]. Unstimulated and stimulated WGA-purified IGF-I receptors (0.25–0.5 μg of total protein in 100 μl of kinase buffer) were mixed with 200 μl of RIPA buffer and 300 μl of binding buffer, then precipitated with 1 μg of GST fusion proteins and 30 μl of glutathione-Sepharose 4B beads. After incubation overnight at 4 °C, samples were washed three times with 1 ml of RIPA buffer. Precipitated samples were boiled for 5 min in loading buffer, fractionated by SDS/PAGE [7.5% (w/v) gel] and transferred to nitrocellulose membranes. The membranes were blocked with 3% (w/v) BSA in PBST buffer [10 mM sodium phosphate (pH 7.2)/140 mM NaCl/0.05% (v/v) Tween-20]. Blots were immunolabelled overnight at 4 °C for phosphotyrosine with monoclonal antibody 4G10 diluted 1:2000, anti-GST polyclonal antibody diluted 1:2000, monoclonal antibody 1.2 to the C-terminus of the IGF-I receptor (1:1000), or polyclonal antibody C-20 to the C-terminus of IGF-I receptor (1:1000; Santa Cruz Biotechnology). Immunolabelling was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL, U.S.A.) in accordance with the manufacturer's directions. Some blots were stripped by incubation for 40 min at 50 °C in a solution containing 62.5 mM Tris (pH 6.7), 2% (w/v) SDS and 0.7% (v/v) 2-mercaptoethanol, then reprobed with other antibodies as indicated in the figure legends.

Phosphorylation of (His)₆-IGF-I receptor fusion peptides *in vitro* and determination of interaction sites between Crk-II and the IGF-I receptor (peptide association assay)

To localize sites required for association of the SH2 domain of Crk-II with the IGF-I receptor, interactions between GST-Crk fusion proteins and phosphorylated (His)₆-fusion peptides representing the JCT domain, the C-terminus and three different tyrosine mutants of the JCT domain (Y943F, Y950F and Y957F) were tested. WGA-purified membrane fractions containing IGF-I receptors (5 μg) were stimulated with 100 nM IGF-I for 40 min in 100 μl of kinase buffer at 22 °C. ATP was added to a final concentration of 0.2 mM and the incubation continued for 15 min. At the end of this incubation (His)₆-peptides (1–4 μg) were added and the incubation was continued for 3–5 h at room temperature. To stop the reaction, EDTA was added to a final concentration of 20 mM. As a control, (His)₆-peptides were mixed with EDTA before the addition of autophosphorylated WGA-purified receptors and the incubation was performed as described. After termination of peptide phosphorylation, 100 μl of RIPA buffer was added and heated for 5 min at 75 °C. After the addition of 2–4 μg of GST fusion proteins and 300 μl of binding buffer with the addition of 0.1% (v/v) non-fat milk, the

mixture was cleared with 15 μ l of Protein A–Sepharose beads, mixed with 30 μ l of glutathione–Sepharose 4B beads and incubated overnight at 4 °C. The precipitates were washed three times with 1 ml RIPA buffer, boiled for 3 min in the loading buffer and loaded on SDS/PAGE [12–15 % (w/v)] gels. After transfer of the proteins to nitrocellulose membrane, the fusion peptides were detected by immunoblotting with an anti-T7-Tag antibody (diluted 1:5000) or an anti-phosphotyrosine antibody (4G10) (diluted 1:2000).

Optimization of (His)₆-fusion peptide purification and peptide association assay

During the purification of (His)₆-fusion peptides and the peptide association assay some technical difficulties arose. Purification of the peptides under native conditions gave a low yield and the purity of the peptides was only 40–50 %. By using denaturing conditions we were able to achieve more than 90 % purity. Both preparations were similarly phosphorylated *in vitro*, demonstrating the suitability of the denaturing conditions.

Some of the peptides were on occasion precipitated from concentrated solutions during dialysis. Neutralization, before dialysis, of the eluates containing the fusion peptides helped to prevent precipitation. Freshly prepared peptides were also partly precipitated from solution during phosphorylation *in vitro*. Incubation of the purified peptide in dialysis buffer [10 mM Tris (pH 8.0)/0.1 % (v/v) Triton X-100/1 % (v/v) glycerol] at 0 °C for 2–3 days helped to prevent precipitation, probably by renaturation of these peptides.

To increase the specificity of the interaction between the GST fusion proteins and phosphorylated peptides, 0.1 % (v/v) non-fat milk was added to the binding buffer as a source of non-specific phosphorylated proteins. In the absence of milk there was a weak interaction of the C-terminal SH3 domain of Crk (but not the N-SH3 domain) with the phosphorylated CT peptide. To prevent co-precipitation of the small amounts of tyrosine-phosphorylated CT and JCT peptides with IGF-I receptor in control experiments when α IR3 antibody (Oncogene Sciences, Uniondale, NY, U.S.A.) was used to immunoprecipitate the IGF-I receptor, the *in vitro* labelling reaction mixture was heated to 75 °C for 5 min after the addition of EDTA and RIPA buffer. The optimal amount of (His)₆-fusion peptides was experimentally determined for phosphorylation *in vitro* and subsequent binding to the GST–SH2 protein. This amount was approx. 0.1 nmol (2.5 μ g) for CT and 0.25 nmol (4 μ g) for JCT peptides. Equimolar amounts of GST–Crk fusion proteins (0.1 nmol) were used in all experiments.

RESULTS

IGF-I receptor phosphorylates Crk-II *in vitro*

Having previously shown that stimulation of cells with IGF-I results in tyrosine phosphorylation of Crk-II in a time- and dose-dependent manner, we were interested in investigating the ability of the IGF-I receptor to phosphorylate Crk-II on tyrosines *in vitro*. To this end, GST–Crk fusion proteins were incubated with immunoprecipitated unstimulated and stimulated IGF-I receptors. Activated IGF-I receptors stimulated the tyrosine phosphorylation of GST–full-length Crk-II in a time-dependent manner (Figure 1A). Interestingly, the unstimulated IGF-I receptors also phosphorylated GST–full-length Crk-II (Figure 1B, lane 1), albeit to a smaller degree than activated receptors, owing to the partial activation of unstimulated receptors in the kinase assay *in vitro* (results not shown). Importantly, neither GST–Crk SH3–SH3 nor GST–Crk SH2 fusion proteins was

tyrosine phosphorylated, despite the addition of equivalent amounts of the fusion proteins to the incubations (Figure 1B). Similar results were obtained with WGA-purified IGF-I receptor (results not shown). Thus it seems that IGF-I receptor-induced tyrosine phosphorylation of Crk-II probably requires the SH2 domain to bind the receptor, and the presence of a tyrosine residue(s) outside the SH2 domain is important for phosphorylation. In separate experiments the GST–SH3–SH3 protein was phosphorylated on tyrosine residue(s) by performing the kinase assay *in vitro* with immunoprecipitated Abl kinase (results not shown).

Crk SH2 domain interacts with the IGF-I receptor from whole cell lysates

Because tyrosine phosphorylation of full-length Crk-II required the presence of the SH2 domain, we wished to determine whether GST–Crk fusion proteins interacted directly with the IGF-I receptor. To this end, cell lysates were obtained from NIH-3T3 cells overexpressing the wild-type IGF-I receptor that had been incubated in the absence or presence of IGF-I. When incubated with GST fusion proteins containing various domains of Crk-II, a significant amount of IGF-I receptor associated only with the Crk SH2 domain itself (Figure 2A; compare lanes 4, 6, 10, 12 and 14 with lane 8). This interaction occurred only with lysates from IGF-I-stimulated cells (Figure 2A; compare lanes 7 and 8). The phosphorylated β subunit of the IGF-I receptor was identified by using an antibody specific to the β subunit of the IGF-I receptor as well as an anti-phosphotyrosine antibody (Figure 2B). GST alone did not associate with the IGF-I receptor (Figure 2A, lanes 1 and 2). The 190 kDa band detected by the anti-phosphotyrosine antibody was found to be the proreceptor because it could also be blotted with antibodies to the β subunit (Figures 2A and 2B, lanes 15 and 16) and α IR3, an antibody directed against the α subunit of the IGF-I receptor (results not shown).

In addition to the tyrosine-phosphorylated β subunit of the IGF-I receptor, two other major tyrosine-phosphorylated proteins could associate with full-length Crk-II as well as with all of the Crk SH2-containing fusion proteins (Figures 2B, lanes 3–8). The approximate molecular masses of these phosphoproteins were 70 and 120–130 kDa. Thus, whereas the IGF-I receptor bound specifically to the GST–SH2 protein only, the pp70 and pp130 phosphoproteins associated with any GST fusion protein containing the SH2 domain. The pp70 corresponded to paxillin [39] as detected with an anti-paxillin antibody (Transduction Laboratories) (results not shown). The phosphoprotein pp130 probably represents a Cas-related protein [40] because antibodies directed against Cas (Transduction Laboratories and Upstate Biotechnology), Cbl (Santa Cruz Biotechnology) and FAK (Transduction Laboratories) did not recognize this protein.

Tyr-221 of Crk-II is phosphorylated by the IGF-I receptor and affects Crk-II-IGF-I receptor interactions

Abl kinase can phosphorylate Crk-II efficiently in intact cells and *in vitro* on Tyr-221, which is located between the two SH3 domains [41]. Once phosphorylated, the SH2 domain of Crk-II can fold and bind at this phosphotyrosine site. This folding of the molecule might affect its interaction with other proteins [41,42]. Despite the fact that the IGF-I receptor phosphorylates Crk-II in intact cells [28] and *in vitro* (see above), we were unable to detect the stable association of these two proteins by co-immunoprecipitation experiments ([28], and results not shown). Thus we postulated that the lack of detection of the interaction of full-length Crk-II with phosphorylated IGF-I receptors in intact cells

Figure 1 Phosphorylation of GST-Crk fusion proteins by activated IGF-I receptors

(A) IGF-I receptors were purified by immunoprecipitation from 200 μ g of total cell lysates from NIH-3T3 cells (NWTb3), overexpressing IGF-I receptors, stimulated with 50 nM of IGF-I for 3 min. The kinase reaction was performed in 200 μ l of kinase buffer containing 2 μ g of GST-Crk II fusion protein. The kinase reaction was initiated by the addition of ATP to a final concentration of 50 μ M; 30 μ l aliquots were taken before initiation (0 min time point) or 5, 10, 15, 20 and 25 min after initiation. The reactions were stopped by the addition of loading buffer. The fusion proteins were fractionated by SDS/PAGE [9% (w/v) gel] and transferred to nitrocellulose membranes as described in the Materials and methods section. Western blot (IB) analysis was performed with the anti-phosphotyrosine (anti PY) antibody (upper panel). The blot was then stripped and reblotted with the anti-GST antibody (lower panel). (B) For each kinase reaction IGF-I receptors were purified by immunoprecipitation from NIH-3T3 cells overexpressing the receptor (NWTb3) as described in the Materials and methods section. Cells were either unstimulated (—) or stimulated (+) with 50 nM IGF-I for 3 min. The kinase reaction was initiated by resuspending the receptor-containing beads in 100 μ l of kinase buffer containing 1 μ g of GST fusion protein and 50 μ M ATP; 30 μ l aliquots were taken after 15 min of the incubation and mixed with loading buffer, and the fusion proteins were separated by SDS/PAGE [9% (w/v) gel]. Transferred proteins were blotted with the anti-phosphotyrosine antibody (upper panel) or, after stripping, with the anti-GST antibody. The results are representative of at least three separate experiments. Abbreviation: c, mixtures with no IGF-I receptor added.

Figure 2 Precipitation of the IGF-I receptor by various GST-Crk fusion proteins

NIH-3T3 cells overexpressing wild-type IGF-I receptors (NWTb3) were either unstimulated (—) or stimulated (+) with 100 nM IGF-I for 2 min. Cells were lysed in RIPA buffer and the lysates (500 μ g of protein) were incubated with equimolar concentrations of various GST-Crk fusion proteins, precipitated with glutathione-Sepharose 4B beads, fractionated by SDS/PAGE [9% (w/v) gel] and transferred to nitrocellulose membrane as described in the Materials and methods section. Western blot (IB) analysis was performed with an antibody against the β subunit of the IGF-I receptor (A). The blot was stripped and reblotted with an anti-phosphotyrosine (anti pY) antibody (B). Lanes 1 and 2, GST alone; lanes 3 and 4, GST-full-length Crk-II; lanes 5 and 6, GST-SH2 SH3; lanes 7 and 8, GST-SH2; lanes 9 and 10, GST-SH3 SH3; lanes 11 and 12, GST-N-SH3; lanes 13 and 14, GST-C-SH3; lanes 15 and 16, immunoprecipitation with α IR3 (Oncogene Sciences Inc, an antibody specific for the α subunit of the IGF-I receptor). The arrow indicates the position of the β subunit of the IGF-I receptor. The results are representative of three separate experiments.

might be due to the rapid tyrosine phosphorylation of Crk-II on Tyr-221 and a subsequent change in folding of the molecule. To test this hypothesis we mutated Tyr-221 to phenylalanine. Activated IGF-I receptors were able to tyrosine-phosphorylate full-length GST-Crk-II (Figure 3A, lane 2), whereas phosphorylation of the mutated Crk-II (Figure 3A, lane 5) and the GST-Crk SH3-SH3 fusion proteins (Figure 3A, lane 8) was decreased significantly. Thus the SH2 domain is required for interaction with the IGF-I receptor and residue 221 is a major tyrosine phosphorylation site *in vitro* for the IGF-I receptor.

When the IGF-I receptor was precipitated by various GST-Crk fusion proteins, the GST-Crk protein with the Tyr-221 replaced with phenylalanine precipitated the IGF-I receptor to a much greater degree than did intact Crk-II. Furthermore the GST-Crk SH2-SH3A fusion protein, which does not include Tyr-221, also precipitated a much greater amount of the IGF-I receptor than the similar construct with Tyr-221 present (Figure 3B; compare lane 2 with 3, and lane 4 with 5). Thus it seems that the presence of the spacer region between the two SH3 domains (residues 208–236) and particularly the presence of Tyr-221, significantly decreases the ability of the SH2 domain of Crk-II to form stable complexes with the IGF-I receptor *in vitro*.

The interaction of the SH2 domain of Crk-II with the IGF-I receptor, as well as with pp70 paxillin and pp130, was completely abrogated by replacement of Arg-38 with valine in the conserved Phe-Leu-Val-Arg-Asp motif (Figure 3B, lane 7). Furthermore no phosphotyrosine-containing proteins were found in the GST-SH3 SH3 precipitates (Figure 3B, lane 8). These findings strongly support the hypothesis that the SH2 domain of Crk-II was solely responsible for the interaction with the IGF-I receptor. In addition it suggests that the binding sites for the Crk SH2 domain on the receptor might possess different properties from similar binding sites on paxillin and p130. Because neither paxillin nor p130 could be seen in GST-p85 SH2 SH2 precipitates (Figure 3B, lane 9), it seems that the properties of the SH2 domains of p85 and Crk-II are also distinct, thus strengthening

Figure 3 Phosphorylation of GST–Crk fusion proteins *in vitro* and the interaction of these proteins with the IGF-I receptor depends on the presence of Tyr-221

(A) The experiment was performed as described in the legend to Figure 1(B). Protein Y221F represents a GST fusion protein consisting of full-length Crk with Tyr-221 replaced by phenylalanine. (B) The experiment was performed as described in the legend to Figure 2. Protein SH2-SH3 A represents the GST fusion protein consisting of the SH2 and N-terminal SH3 domain of Crk-II excluding Tyr-221 (residues 9–208). Protein SH2-SH3 represents the SH2 and N-terminal SH3 domain of Crk including Tyr-221 (residues 9–236). Co-precipitated proteins were analysed with the anti-IGF-I receptor β subunit antibody (upper panel), stripped and reblotted with the anti-phosphotyrosine antibody (lower panel). These results are representative of three separate experiments. The arrowhead and arrow indicate the positions of the IGF-I receptor β subunit and pp130 respectively. Abbreviations: IB, immunoblot; anti PY, antiphosphotyrosine.

the suggestion that the interaction of Crk-II with the IGF-I receptor is specific.

Specificity of the interaction of the Crk-SH2 domain with the IGF-I receptor

To establish that the interaction of the SH2 domain of Crk-II with the IGF-I receptor was specific, we tested various SH2 domains from other proteins. It has been shown that PLC γ , containing two SH2 domains, does not bind the IGF-I receptor, whereas the p85 regulatory subunit of PtdIns 3'-kinase, containing two SH2 domains, does interact with the IGF-I receptor *in vitro* [25]. To determine whether the interaction of the SH2 domain of Crk-II with the IGF-I receptor is specific, we compared the binding ability of the GST–Crk SH2 fusion protein with those containing either the SH2 domain of PLC γ (separately) or the SH2 domains of the p85 subunit of PtdIns 3'-kinase. When GST fusion proteins from these various proteins were incubated with WGA-purified and activated IGF-I receptors, both Crk-II and p85 SH2 domains precipitated the IGF-I receptor, whereas neither the N-terminal nor the C-terminal SH2 domain of PLC γ was capable of doing so (Figure 4A, lanes 1–8). Similarly, GST fusion proteins encoding the SH2 domains of PLC γ were unable to co-precipitate activated IGF-I receptors from total cell lysates (results not shown). Furthermore, when the specific arginine residue within the Phe-Leu-Val-Arg-Asp sequence responsible for the interaction with phosphorylated tyrosine residues of the Crk-SH2 domain was mutated (GST–SH2 RV fusion protein), the interaction with the IGF-I receptor was abrogated (Figure 4A, lanes 9–12).

Crk-SH2 domain interacts preferentially with the JCT domain of the IGF-I receptor

To specify the interaction site(s) of the IGF-I receptor with the SH2 domain of Crk-II we expressed the JCT and CT domains of the receptor as (His)₆-tagged peptides, phosphorylated the peptides *in vitro* and tested them for interaction with different GST fusion proteins encoding the SH2 domains of Crk-II, PLC γ and

the p85 subunit of PtdIns 3'-kinase (Figure 4B). Under these conditions the SH2 domain of Crk-II interacted preferentially with the JCT peptide, in contrast with the interaction with the CT peptide. Conversely, the p85-SH2 domains showed a stronger association with the CT peptide. This result is consistent with their known interactions with the CT domain of the IGF-I receptor [25]. Similarly to the findings with the full-length IGF-I receptor, no interaction was seen between the JCT and CT peptides and the SH2 domain of PLC γ .

To characterize further the interaction of the JCT peptide with Crk-II, we tested the ability of various GST–Crk proteins to interact with the JCT peptide (Figure 5). Only those fusion proteins containing the SH2 domain interacted with the JCT peptide (Figure 5A, lanes 2, 3 and 4), and the strongest interaction was with Crk SH2 alone (lane 4). Analysis of the supernatants from the same reactions (Figure 5B) showed the presence of two bands containing tyrosine-phosphorylated residues, possibly indicating the presence of two phosphorylated tyrosines. Interestingly, only the more slowly migrating peptides were able to form stable complexes with the GST–Crk SH2 fusion proteins, indicating that more than one phosphotyrosine is necessary to form these complexes.

To determine which tyrosines in the JCT domain were necessary for this effect, each tyrosine (Tyr-943, Tyr-950 and Tyr-957) in the JCT peptide was mutated to phenylalanine, and *E. coli*-expressed peptides were analysed for their ability to bind the GST–Crk SH2 protein. Results of this experiment are presented in Figure 6. Phosphorylation of the JCT peptide is absolutely necessary for its interaction with the SH2 domain of Crk-II because no interaction was found when the tyrosine kinase activity of WGA-purified IGF-I receptors was inhibited by EDTA (Figures 6A and 6B; compare lanes 1 and 2). Replacement of Tyr-957 by phenylalanine did not change the ability of the JCT peptide to be tyrosine phosphorylated *in vitro* (Figure 6A; compare lanes 7 and 10) and only decreased the association of the JCT peptide with the GST–Crk SH2 protein. In contrast, mutations of Tyr-943 or Tyr-950 significantly decreased the ability of the JCT peptide to be phosphorylated on tyrosine residues (Figure 6A, lanes 8 and 9) and completely abrogated the

Figure 4 Specificity of the interaction of the Crk-SH2 domain with the IGF-I receptor

Various SH2 domain-containing GST fusion proteins (2 μ g of each) were incubated with increasing amounts of WGA-purified IGF-I receptors (250 or 500 ng) that were activated *in vitro* as described in the Materials and methods section. The GST fusion protein-receptor complexes were then collected by using glutathione-Sepharose 4B beads and the precipitated IGF-I receptors were detected by immunoblotting with the anti-phosphotyrosine antibody. **(A)** GST fusion proteins of Crk-SH2 (lanes 1, 2, 11 and 12), C-terminal SH2 domain of PLC γ (lanes 3 and 4), SH2-SH2 domains of p85 (lanes 5 and 6) and N-terminal SH2 of PLC γ (lanes 7 and 8); SH2 RV represents the SH2 domain of Crk-II with a replacement of Arg-38 with Val (lanes 9 and 10). **(B)** JCT and CT peptides were phosphorylated *in vitro* with WGA-purified autophosphorylated IGF-I receptors (+). EDTA chelation (–) was used to inhibit the phosphorylation of the peptides. JCT and CT peptides were incubated with GST fusion proteins consisting of the SH2 domain of Crk-II (lanes 1–4), C-terminal SH2 domain of PLC γ (lanes 5 and 6), SH2-SH2 domains of p85 (lanes 7 and 8) or the N-terminal SH2 domain of PLC γ (lanes 9 and 10). After precipitation with glutathione-Sepharose 4B beads, bound JCT and CT peptides were detected by immunoblotting using an anti-phosphotyrosine antibody. The results are representative of two separate experiments.

interaction of GST-Crk SH2 with the tyrosine-phosphorylated JCT peptide (Figure 6A, lanes 3 and 4). The presence of non-tyrosine-phosphorylated Tyr-943, but not Tyr-950, in GST-Crk SH2 precipitates (Figure 6B, lanes 3 and 4) indicates that other modifications might have some role in this association. On the basis of the results in Figures 5 and 6 we conclude that Tyr-943 and Tyr-950 seem to be the residues primarily tyrosine phosphorylated and involved in the interaction of the JCT peptide

Figure 5 Interaction of various GST-Crk fusion proteins with the JCT peptide

A peptide from the JCT region of the IGF-I receptor was phosphorylated with WGA-purified IGF-I receptors. The phosphorylated JCT peptide was incubated with various GST-Crk fusion proteins and precipitated as described in the Materials and methods section. **(A)** Proteins were transferred to nitrocellulose membranes and immunoblotted with the anti-phosphotyrosine antibody. **(B)** The supernatants from each reaction, immunoblotted with the anti-phosphotyrosine antibody. Lane 1, GST alone, lane 2, GST-full-length Crk, lane 3, GST-SH2 SH3, lane 4, GST-SH2, lane 5, GST-SH3 SH3, lane 6, GST-N-SH3; lane 7, GST-C-SH3. The arrows represent the tyrosine-phosphorylated JCT peptides. The results are representative of two separate experiments.

Figure 6 Interaction of various mutated JCT peptides of the IGF-I receptor with the GST-Crk SH2 fusion protein

Mutated or wild-type JCT peptides were phosphorylated with WGA-purified IGF-I receptors either in the absence (+, stimulated) or presence (–, unstimulated) of EDTA, incubated with the GST-Crk SH2 fusion protein and precipitated as described in the Materials and methods section. After SDS/PAGE the (His) $_6$ -JCT peptides were detected by immunoblotting (IB) with the anti-phosphotyrosine (anti pY) antibody **(A)**. The blots were stripped and reprobed with the anti-T7 antibody **(Panel B)**. Lanes 1–5 represent proteins co-precipitated with GST-Crk SH2 fusion protein. Lanes 6–10 represent proteins remaining in the supernatant after precipitation. Lanes 1, 2, 6 and 7, wild-type JCT peptide; lanes 3 and 8, Y943F (mutation of Tyr-943 to phenylalanine); lanes 4 and 9, Y950F (mutation of Tyr-950); lanes 5 and 10, Y957F (mutation of Tyr-957). The results are representative of three independent experiments.

Figure 7 Interaction of GST-SH2 fusion proteins with the JCT region of the IGF-I receptor

NIH-3T3 cells overexpressing wild-type receptors and mutant (Tyr-950) receptors were stimulated by 50 nM IGF-I for 3 min. Lysates were prepared and incubated with GST-SH2 fusion proteins. The complex of receptor and GST fusion protein was collected by using glutathione-Sepharose 4B beads and the receptor was detected by immunoblotting with an anti-phosphotyrosine antibody. Lanes 1 and 2, 30 µg of total cell lysates from the Y950F.3 cell line overexpressing the mutated IGF-I receptor where Tyr-950 was substituted with phenylalanine; lanes 3 and 4, precipitation of the stimulated IGF-I receptor with GST-SH2 SH2 of the p85 subunit of Ptdins 3'-kinase; lanes 5 and 6, precipitation of the stimulated IGF-I receptor with GST-Crk SH2. Abbreviations: b3, cell lysates prepared from the NWTb3 cell line; 950, cell lysates prepared from the Y950F.3 cell line. These results are representative of three independent experiments.

with the Crk SH2 domain. No interaction of the JCT peptide was found with GST-Crk SH2 Arg-38 → Val substitution (results not shown).

To confirm the importance of Tyr-950 of the IGF-I receptor for the direct binding of Crk-II to the receptor, we overexpressed the Y950F mutant receptor in NIH3T3 cells and compared the ability of wild-type and mutant receptors to associate with the SH2 domain of Crk-II *in vitro*. Figure 7 illustrates the results of such experiments. Mutation of Tyr-950 does not affect receptor autophosphorylation (Figure 7, lanes 1 and 2). Furthermore it does not affect the ability of the SH2 domains of p85 to bind the receptor (Figure 7, lanes 3 and 4). The increased amount of phosphorylated β subunit in lane 4 reflects the increased number of mutant receptors expressed in the Y950F.3 cell line. Most significant is the marked decrease in the ability of the SH2 domain of Crk-II to form stable complexes with the mutant receptor (Figure 7, lanes 5 and 6). Six independent clones expressing different numbers of the Y950F receptors were similarly tested, all of which demonstrated significant decreases (to one-fifth or one-sixth) in the ability of the SH2 domain to bind the mutated IGF-I receptors (results not shown).

DISCUSSION

The role of the Crk family of proteins in signalling and cellular function has been demonstrated by cellular overexpression of Crk. The stable expression of v-Crk in PC12 pheochromocytoma cells results in accelerated differentiation as assessed by the induction of neurites after treatment with nerve growth factor (NGF) or basic fibroblast growth factor compared with the effect in native PC12 cells [43]. However, these cells also develop extensive neurite processes after stimulation with epidermal growth factor (EGF), an event that is not observed in native PC12 cells. Expression of v-Crk results in an enhanced and sustained activation of Ras and MAP kinase after stimulation

with EGF or NGF, implying that v-Crk can couple divergent tyrosine kinase pathways to Ras [44]. Overexpression of wild-type Crk-II, the cellular analogue of oncogenic v-Crk, in PC12 cells enhanced the NGF-induced activation of Ras, although the basal level of GTP-bound active Ras was not altered [29]. In contrast, mutants with a single amino acid substitution in either the SH2 or SH3 domains of the Crk protein inhibited the NGF-induced activation of Ras [34]. Two guanine nucleotide-releasing proteins, C3G and mSOS, interact with the N-terminal SH3 domain of Crk in a growth factor-independent manner [35]. This interaction might regulate Ras, thus triggering downstream effectors in the Ras/Raf-1/MAP kinase pathway.

Results of Gotoh et al. [45] indicate that C3G is an activator of Rap1/Smg p21/Krev-1, which interferes with the Ras signalling pathway. Thus the adaptor protein Crk-II might have a crucial role in cell growth and differentiation via these proteins. The recent finding that a dominant negative mutant of Crk-II interrupts signalling from the EGF receptor to Ras and prevents the oncogenic transformation of rat fibroblasts supports this hypothesis [46]. IGF-I stimulation of NIH-3T3 and 293 cells induces the tyrosine phosphorylation of Crk-II in a time- and dose-dependent manner, indicating that Crk-II might be an endogenous substrate for the IGF-I receptor. Furthermore the overexpression of Crk-II enhances the mitogenic response of NIH-3T3 cells to IGF-I, providing additional evidence of the role of Crk-II in IGF-I receptor signalling [28,36].

Our results demonstrate that the activated IGF-I receptor efficiently phosphorylates Crk-II *in vitro* in a time-dependent manner, supporting the idea that Crk-II might be a direct substrate of the receptor in intact cells. Furthermore this phosphorylation event depends on the presence of the SH2 domain of Crk-II and the presence of Tyr-221. Thus we suggest that the SH2 domain of Crk-II binds the activated IGF-I receptor, and the tyrosine kinase of the IGF-I receptor phosphorylates Tyr-221 of Crk-II. It has been previously shown that Tyr-221 of Crk-II is a major phosphorylation site for Abl kinase [41]; most interestingly, after phosphorylation, the SH2 domain of Crk-II binds this site. This intramolecular event might decrease the ability of Crk-II to bind other proteins [41,42]. On the basis of these observations, we propose a mechanism of direct but transient interaction of Crk-II with the IGF-I receptor. On stimulation with IGF-I and autophosphorylation of the receptor, Crk-II binds directly to the receptor via its SH2 domain. The tyrosine kinase of the receptor phosphorylates Tyr-221 of Crk-II, which then causes the dissociation of Crk-II from the receptor owing to the intramolecular conformational change of the Crk-II molecule. Such a mechanism might explain the inability to detect the interaction in intact cells with co-immunoprecipitation techniques. Formation of this intramolecular structure on Crk-II phosphorylation implies that the SH2 domain of Crk-II binds phosphorylated IGF-I receptors with relatively low affinity. As an indicator of this, the GST-SH2 protein precipitates significant amounts of the IGF-I receptor, whereas GST-SH2 SH3 and full-length Crk-II exhibit very low levels of association. This is reminiscent of the interaction of Crk-II with the NGF receptor, TrkA [29,44]. In contrast, paxillin and p130 bind all three fusion proteins with similar affinities, indicating that the binding interactions for Crk-II of these proteins differ from that of the IGF-I receptor. We suggest that the decreased ability of GST-SH2 SH3 and full-length Crk-II to recognize the low-affinity binding sites on the IGF-I receptor and TrkA, under the conditions of the binding assays *in vitro*, is due at least in part to the folding of bacterially expressed fusion proteins. Removal of the spacer region between the two SH3 domains (residues 208–236) or the point mutation of Tyr-221 significantly increases

the ability of those fusion proteins to bind the activated IGF-I receptor *in vitro*.

The Crk SH2 domain has been predicted to have a high affinity for phosphopeptides with an aspartic residue in position +1 (relative to the phosphotyrosine residue) and, more importantly, a proline residue in the +3 position [47]. It has been shown that the interaction of Crk-II with paxillin [48], the EGF receptor [49], Cbl [50] and p130 Cas [51] might occur through a phosphotyrosine residue with a proline residue in position +3. However, neither the IGF-I receptor nor TrkA [52] possesses this sequence. To determine the binding site of the SH2 domain of Crk-II on the IGF-I receptor we developed an *in vitro* system to measure the ability of (His)₆-fusion peptides phosphorylated *in vitro*, containing different domains of the IGF-I receptor, to associate with GST-Crk fusion proteins. We show that this association mimics the interaction between GST-Crk proteins and the full-length IGF-I receptor to a significant degree. For example, only phosphorylated peptides are able to associate with GST fusion proteins containing the SH2 domain of Crk-II, suggesting that the IGF-I receptor must be stimulated and phosphorylated before association with Crk-II can occur. Similarly to the native IGF-I receptor, the affinity of the GST-SH2 protein for these phosphorylated peptides is higher than the affinity for GST-full-length Crk. The specificity of this interaction is demonstrated by the use of other SH2 domains. The phosphorylated peptides from the IGF-I receptor do not bind the SH2 domains of PLC γ , whereas the SH2 domains of p85 bind the CT domain of the IGF-I receptor much more strongly than the JCT domain. These findings confirm the results of previous studies involving the full-length IGF-I receptor [25].

We chose to study the interaction of Crk-SH2 with both the JCT and CT domains, because we have found that GST-Crk SH2 co-precipitates 50-fold more IGF-I receptors from cell lysates than insulin receptors (results not shown). The JCT and CT domains are the least conserved regions in the cytoplasmic portion of these two otherwise closely related receptors. The results of our peptide association assay demonstrate that the SH2 domain of Crk-II preferentially binds the JCT region of the IGF-I receptor. By using mutated peptides, we show that the association of the GST-SH2 fusion proteins with phosphorylated JCT peptide seems to depend on the presence of two tyrosine residues, Tyr-943 and Tyr-950, because mutating either residue significantly impairs (Tyr-943) or completely abrogates (Tyr-950) the interaction of the proteins. From the results presented here it is not clear whether the absence of association of Y943F and Y950F peptides with GST-Crk SH2 is due to the necessity of the SH2 domain to bind phosphorylated Tyr-943 and Tyr-950 simultaneously, or whether both tyrosine residues are required for the proper conformation of JCT peptide, allowing one of the residues to be phosphorylated and participate in association with the SH2 domain of Crk-II. In support of the latter proposition, Prager et al. [53] showed that all of the juxtamembrane substitutions of IGF-I receptor from Gly-940 to Tyr-957 significantly impaired the internalization rate of ¹²⁵I-labelled IGF-I, demonstrating that the overall protein structure of the JCT domain was important for the proper functioning of the receptor. Nevertheless we have confirmed the results of our peptide association assay, by showing that the Tyr-950 mutation significantly decreases the ability of GST-Crk SH2 to co-precipitate the IGF-I receptor from whole-cell lysates. Preliminary experiments have demonstrated that overexpression of Crk-II in 293 cells caused a decrease in the IGF-I-induced tyrosine phosphorylation of IRS-1 in comparison with parental cells. We interpret this finding as evidence that Crk-II might compete with IRS-1 for binding to the same site in the JCT domain of the IGF-

I receptor (results not shown). Comparisons of the residues of the IGF-I receptor and TrkA show that the residues surrounding Tyr-950 of the IGF-I receptor are highly similar to the region surrounding Tyr-490 in the JCT region of TrkA. Comparison of ten amino acids (−3 to +6, with respect to the tyrosine residues) revealed that eight out of ten were identical [52]. Taking into consideration the similar properties of these two receptors with regard to Crk-II, we speculate that Tyr-490 of TrkA might be the binding site for Crk-II. So far no results are available on the phosphorylation sites of the JCT and CT domains of the IGF-I receptor in intact cells. Our results from the studies *in vitro* show that Tyr-943 and Tyr-950 are the major sites of phosphorylation in the JCT peptide, with Tyr-957 showing minimal phosphorylation. This is similar to the phosphorylation of the insulin receptor, where the equivalent Tyr-953 and Tyr-960, but not Tyr-972, are phosphorylated [54], with Tyr-960 being the most phosphorylated. Additionally, phosphoserine residues have been identified in the JCT region after insulin receptor activation in intact cells [54,55]. We suggest that this and/or additional modification(s) of the JCT region of the insulin receptor might decrease the ability of the GST-Crk SH2 protein to co-precipitate this receptor from total cell lysates. Furthermore the differences in the structure of the CT domains of the IGF-I and insulin receptor might contribute to the differential binding. Crk-II might also interact with Shc [29] and with IRS-1 and IRS-2 [36] suggesting that, in addition to a direct interaction with the IGF-I receptor, there might be other mechanisms for the phosphorylation of Crk-II by the activated IGF-I receptor. The role of each of these proteins in IGF-I-dependent phosphorylation of Crk-II needs to be determined.

In summary, by using studies *in vitro* we have demonstrated that the IGF-I receptor effectively phosphorylates Crk-II *in vitro* on Tyr-221. This phosphorylation was dependent on the presence of the SH2 domain of Crk-II, suggesting a direct interaction of these two proteins. With a peptide association assay, we identified Tyr-950 of the JCT of the IGF-I receptor as the major phosphotyrosine responsible for this interaction. On the basis of these studies *in vitro*, a model for the interaction of the IGF-I receptor and Crk-II *in vivo* is proposed, in which the interaction is direct although transient.

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